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FROM THE STATE SERUM INSTITUTE, HELSINKI

CORD FACTOR AS ANTIGEN IN COMPLEMENT FIXATION AND FLOCCULATION TESTS

by

VEIKKO TOMMILA and HELGE HONKAPOHJA

(Received for publication January 19, 1954)

The cord structure present in the colonies of pathogenic tubercle bacillus strains, to which attention was paid by Middlebrook, Dubos and Pierce (4), is due, according to Bloch (1, 2), to a special connective substance the bacteria secrete around themselves. Bloch isolated this so-called cord factor by means of short petrol ether extraction. The factor is a wax-like substance with a low melting point, containing no phosphor and is not acid-fast on staining. Petrol ether extraction from bacterial culture does not, according to Bloch, greatly disturb the bacterial viability and growth, though it perceptibly reduces the virulence of the strain, and the cord structure in the colonies is broken. Later, Bloch, *et al.* (3), showed that the cord factor consists of several different components.

As, according to Bloch's observations, the cord factor is a secretion of young, rapidly multiplying bacteria and covers the bacterial surface as the outermost layer, it seems possible that the organism produces antibodies especially against a bacterial derivative resembling a surface antigen. This possibility led the authors to study the cord factor as an antigen in complement fixation and flocculation tests.

METHODS

1. *Cord Factor.* — The cord factor [1] was prepared from the two-week-old surface membranes of Strain H 37Rv grown on Sauton medium, in the main

[1] The cord factor employed in the present investigation was prepared from a petrol ether fraction of tubercle bacilli received from Prof. H. G. Haahti.

according to the method of Bloch but with the following modifications: (*a*) Extraction was effected by shaking the bacterial mass with steel balls in a thick-walled glass flask. (*b*) Centrifugation was carried out in an ordinary centrifuge (3,500 rpm). (*c*) The petrol ether was evaporated in a vacuum flask without using nitrogen atmosphere by admitting petrol ether into the flask through a very thin capillary in small quantities that evaporated rapidly. (*d*) The white mass precipitated three times by alcohol from the ether solution was finally dissolved in dioxane.

2. *Antigens.* — Antigen I: Antigen suspension was prepared from the cord factor with cholesterol and lecithin by the technique employed in the VDRL syphilis reaction (4). Three hundredths per cent of cord factor, 0.9 per cent of cholesterol and 0.3 per cent of lecithin gave the »silkenswirl» suitable for antigen suspension.

Antigen II: The tuberculin fraction employed in the Middlebrook-Dubos haemagglutination test was added to Antigen I by substituting tuberculin fraction for one-fifth of the buffer (0.4 cc) used in the preparation of one antigen suspension dose. This ratio proved optimal.

Antigen III: For the Middlebrook-Dubos haemagglutination test and its haemolytic modification (Middlebrook), Sauton's tuberculin was precipitated at a low temperature with 96 per cent ethyl alcohol; the precipitate was dried and dissolved in buffered physiological saline solution. The strength of the antigen was adjusted to equal that of Lederle's tuberculin »4 × standard».

3. *Tests.* — (*a*) The complement fixation test was carried out in a total volume of 0.5 cc. A 1 : 3 dilution of the inactivated (56° C, 30 min.) sera was employed. The complement was titrated with the use of various amboceptor dilutions in parallel. A 1.3-fold amount of complement was used in the tests. For fixation, the tubes were first kept for 45 min. at room temperature and then for 30 min. at +37° C. Readings were taken after approximately 15 and 30 min. The tests in which no or only a slight haemolysis occurred were considered positive (+). In the event of a partial haemolysis, the reaction was considered weakly positive (±).

(*b*) The slide flocculation test was effected in the same way as the VDRL syphilis slide test. A definite flocculation of antigen particles was considered to indicate a positive reaction, (+) and a weak flocculation a weakly positive (±) result.

(*c*) The Middlebrook-Dubos (6) haemagglutination test and the Middlebrook (5) haemolytic modification were carried out in the way described by these authors. The sera, however, were absorbed over night with sheep's red cells (0.2 cc of 100 per cent cells to 1 cc of serum). If in agglutination test the red cell agglutination was to be seen in a serum dilution $\geq 1 : 16$ and in the haemolytic modification the haemolysis in the serum dilution 1 : 2 was $\geq$ 50 per cent, the result was considered positive.

RESULTS

The sera from a total of 177 sanatorium patients with active pulmonary tuberculosis and from 50 healthy blood donors were investigated. Of the former, 75 sera were examined with the complement fixation test using Antigen I (cord factor) and 102 sera with Antigen II (cord factor + tuberculin precipitate). The sera of the blood donors were examined with both the cord factor antigens. A number of the sera of the tuberculous patients and all the sera of the healthy subjects were examined with the slide flocculation test using both Antigen I and II. The control reactions consisted of the Middlebrook-Dubos haemagglutination test and the Middlebrook haemolytic modification of the former, with which all the sera were examined.

Table 1 shows the results obtained with cord factor antigens in the complement fixation test and in the Middlebrook-Dubos (M—D) haemagglutination test and its haemolytic modification (M).

TABLE 1

	Number of Sera	Antigen I			Antigen II			M-D	M
		+ %	± %	Total	+ %	± %	Total	+ %	+ %
Active pulmonary tuberculosis patients	75	8	17	25				83	83
	102				28	14	42	54	75
Healthy subjects	50	0	2	2	0	2	2	14	12

With Antigen I, 8 per cent of the sera of 75 tuberculous patients gave a positive reaction to the complement fixation test. If the weakly positive results are included, the percentage of positive reactions, increases to 25. Both of the control tests had a positive result in 83 per cent. With Antigen II, the results were 28 and 42 per cent, respectively. Thus, the tuberculin precipitate considerably increased the sensitivity of the antigen, as is indicated by the larger number of positive results, although the control tests gave considerably fewer positive results in this group than in the former group.

None of the sera of the blood donors reacted positively to either of the cord factor antigens, though one ± result was obtained with both. In this control group, among the control reactions, Middle-

brook's haemolytic modification revealed a somewhat higher specificity than the Middlebrook-Dubos haemagglutination test. The results obtained with the last-mentioned tests comply with those reported in the literature.

The positive results obtained with the slide flocculation test were considerably fewer than with the complement fixation test. With Antigen I, 5 per cent of the sera of 56 tuberculous patients reacted positively. Including weakly positive reactions, the result was 9 per cent. The corresponding percentages for 84 sera with Antigen II were 8 and 30; hence, in this case also the addition of tuberculin fraction increased the sensitivity of the antigen. Not a single serum of the healthy subjects gave a positive result with any of the antigens. The positive results yielded by the slide flocculation test were obtained in only approximately half the cases with sera that reacted positively to the complement fixation test.

A few positive sera were titrated to ascertain the degree of the positivity of the sera. Generally speaking, the titres were low (1 : 3). The greatest serum dilution that gave a positive result with the complement fixation test was 1 : 200 (Antigen I) and with the flocculation test 1: 16 (Antigen II).

With cord factor positive sera, the titres of the Middlebrook-Dubos and the Middlebrook reactions varied evenly from 1 : 1 to 1 : 64. In some cases the control tests gave a negative result with cord factor positive (complement fixation) sera. All the sera that reacted positively to Antigen I gave a positive reaction in the control tests also, whereas six of the sera that gave a $\pm$ result with Antigen I reacted negatively in the Middlebrook-Dubos test and two in the haemolytic test. One of them was negative in both control reactions. Of the sera positive to Antigen II, nine reacted negatively in the Middlebrook-Dubos test, three to the haemolytic test and one in both tests. Of the weakly positive sera, three gave a negative result in the Middlebrook-Dubos test. Hence the cord factor complement fixation tests (Antigens I and II) somewhat increased the number of positive results obtained with the control reactions.

The tuberculous patients from whom the sera under investigation had been taken had active pulmonary tuberculosis of varying severity and duration. No definite difference in the extent and nature of pulmonary changes or in the general condition was

observable between patients whose sera reacted cord factor positively and those whose sera reacted negatively. No particular difference from the total series could be observed with the two patients whose sera reacted very strongly positively in the complement fixation test (titres 1 : 150 and 1 : 200).

DISCUSSION

From the investigation, it seems that, as a fairly low percentage of the sera of tuberculous patients reacts positively to both cord factor and combined antigen, the reaction in this form if used alone has no practical importance in the diagnosis of pulmonary tuberculosis. On the other hand, the complement fixation test could possibly be employed together with the Middlebrook-Dubos haemagglutination test and its haemolytic modification, which are so far largely unspecific, particularly since the *positive reactions to the complement fixation test with the control material were almost non-existent.* But it alone gave two positive results on tuberculosis sera. The antigen to which tuberculin precipitate has been added, because of its higher sensitivity, is preferable to cord factor antigen alone. Evidently this reaction could be made more sensitive still, but in that case an increase in potential false reactions must be considered. The results obtained in the present investigation support the opinion that, owing to the obvious antigenic heterogenicity of tubercle bacilli and the evidently varying ability of different individuals to form antibodies against these various components of the bacilli, in the serodiagnosis of tuberculosis efforts should be made to employ tests as varying as possible with the use of different fractions of tubercle bacilli as antigens.

SUMMARY

Using as antigen the cord factor formed by tubercle bacilli with cholesterol and lecithin, either alone or together with tuberculin precipitate, in complement fixation and, partly, in slide flocculation tests, the sera from a total of 177 patients with active pulmonary tuberculosis and those from 50 healthy blood donors were investigated. For control, the sera were also examined with the Middlebrook-Dubos haemagglutination test and its haemolytic modification. Of the sera from tuberculous patients, 8 and 28 per

cent reacted positively in the complement fixation test. If the weakly positive reactions are also included, the figures are 25 and 42 per cent, respectively. Combined antigen gave higher percentages. Considerably fewer positive results were obtained with the flocculation test. In the control group a single serum gave a weakly positive reaction. The results obtained with the control tests complied with the results reported in the literature. If the complement fixation test is used alone, the reaction has no practical significance in the diagnostics of pulmonary tuberculosis, but together with the control tests the complement fixation test might provide a diagnostic aid.

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FROM THE STATE SERUM INSTITUTE AND THE FINNISH RED CROSS BLOOD SERVICE, HELSINKI

THE PREPARATION OF NORMAL HUMAN SERUM FOR INTRAVENOUS USE

by

EVA ALAMERI

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The normal human blood plasma in use in Finnish hospitals as a blood volume expander is prepared from ACD blood. This blood contains a solution of citric acid, sodium citrate and dextrose as an anticoagulant and red cell preservative. The plasma is prepared from the blood according to the following procedure:

The plasma and the red cells are separated, and calcium ions are added to the plasma. The process of coagulation is thus allowed to proceed. During coagulation the plasma is stirred; the fibrin formed is then removed as a solid mass, and 5 per cent dextrose is added as a stabilizer to the remaining liquid, which contains the bulk of the plasma proteins. The liquid is finally clarified and sterilized by filtration. This method gives a clear »defibrinated plasma» or serum. Those osmotic properties of this serum that depend on the proteins remain practically unchanged for a long period, even when the serum is stored at room temperature.

The value of a serum thus prepared is, however, reduced by the presence of a heavy white calcium citrate precipitate, which separates from the serum during the first two to eight weeks of storage. In a solution rich with calcium and citrate ions, the calcium citrate complex slowly turns to an insoluble form when the proteins bind some of the complex water.

In order to eliminate this disadvantage, the possibilities of preparing a serum free of precipitable substances have been studied.

MATERIAL AND METHODS

The anion exchange resin, Amberlite IRA 400 (Rohm & Haas Co., Philadelphia, Pa., U.S.A.) was freed from pyrogenous matter and brought to chloride cycle by treatment with 10 N NaOH, 1 N HCl, pyrogen-free water and 0.15 M NaCl, in that order.

The cation exchange resin, Dowex 50 (Dow Chemical Co., Jersey City, N.J., U.S.A.), was freed from pyrogenous matter and brought to sodium cycle by treatment with 1 N HCl, 10 N NaCl, pyrogen-free water and 0.15 M NaCl, in that order.

One bottle of ACD blood contains ca. 400 ml of blood and 75 ml of ACD solution, consisting of 4 g of citric acid, 14 g of sodium citrate and 22 g of dextrose in 1 liter. The plasma, ca. 200 ml, separated from the red cells thus contains ca. 1.2 g or 10.8 meq. of citrate. If the capacity of the anion exchange resin is 0.7 (80 per cent saturated and moist Amberlite IRA 400), the amount needed to bind the citrate is thus 15.3 g of resin per 200 ml of plasma.

Of the ethylenediaminetetraacetic acid complexes, the disodium form EDTA Na2 (P. H. Tamm, Altuna, Sweden) was used, 75 ml of a solution containing 11 g of EDTA Na2 and 50 g of dextrose per liter being used to each bottle of 400 ml of blood.

Calcium was added in the form of a 20 per cent $CaCl_2$ solution.

The content of EDTA Na2 complex in the serum was determined according to Darbey (2). The protein from the samples and controls was precipitated with trichloroacetic acid prior to the determination.

The pyrogenicity of the serum was determined according to the method given in the *United States Pharmacopeia*.

Protein concentration measurements were made with a Beckman model DU spectrophotometer at 280 mμ. The extinction value $E\,{}^{1\ \%}_{1\ cm} = 10$ was used for serum.

RESULTS

If ACD blood is desired as raw material in serum preparation, the ion exchange resins can be used. The citrate ions are removed from the plasma that has been separated from the red cells by exchanging them to chloride ions. The calcium is thus liberated from the citrate complex, with the exception of the amount which is bound to the resin together with citrate, and the coagulation can again continue. The exchange can be performed by running the plasma through a resin column or by mixing the plasma with the resin. The serum is decanted from the fibrin-resin mass after coagulation. The amount of resin needed in the reaction can be

calculated when the citrate concentration of the plasma and the capacity of the resin are known. The reaction requires more than the theoretical amount of resin when done batchwise, because the reaction settles in equilibrium according to the concentrations of the ions in the solution. The resin can be cleaned with a strong sodium hydroxide solution and regenerated.

The rest of the procedure is similar to that described in the introduction. The result is a serum which is equal to the calcium citrate serum, except that it contains no citrate ions and no excess of calcium. The protein concentration is ca. 5 per cent. The serum meets the requirements of the pyrogen test. On storage, it remains clear and its ionic concentration is unchanged.

However, the use of ion exchange resins in the preparation of large quantities of serum is laborious, and another method for preparing clear serum has, therefore, been studied. This method is based on the binding of the blood calcium with a compound that gives more soluble calcium salts than citrate yields. Such a compound should also be indifferent to red cells, nontoxic in the concentrations to be used, noncumulative and stable on autoclave sterilization in solution. The disodium salt of ethylenediaminetetraacetic acid meets these requirements. It was first used in the analytical chemistry of metals but has aroused interest also as an anticoagulant (3, 4, 5, 8). A volume of 100 ml of water at 30° C dissolves 0.25 g calcium citrate = 0.0044 M = 0.013 eq. calcium or 4.46 g EDTA Na2 Ca = 0.1 M = 0.1 eq. calcium (1).

If instead of the ACD solution, an EDTA Na2 solution is used as an anticoagulant and red cell preservative in the blood bottle, the preparation of serum can be carried out by the method described in the introduction. More calcium ions than the EDTA Na2 is capable of binding are first added to the plasma. The equivalence point can be calculated or determined by titration. The plasma is then stirred until the fibrin can be separated. After the addition of 5 per cent dextrose the serum is clarified and sterilized by filtration. The serum when ready for infusion contains ca. 1 g of soluble EDTA Na2 Ca per 500 ml of serum.

In this method the amount of EDTA Na2 Ca in serum can also be decreased by using ion exchange resins in smaller amounts. If some amount of anion exchange resin in chloride cycle or of cation exchange resin in sodium cycle, or of both, is mixed with

the serum before filtration, the amount of EDTA complex is decreased by a corresponding amount.

The *p*H of serum prepared in this manner is 6.8—7.2. The protein concentration is 5.0—5.5 per cent. The serum meets the requirements of the pyrogen test, if all the solutions and vessels that are used in the preparation are pyrogen-free. The serum remains clear, and its water-binding capacity is unchanged for 25 to 50 weeks, *i.e.*, until the labile proteins begin to cause turbidity.

A plasma or serum used as infusion liquid should be independent of the blood groups. With the methods that have been described above, serum independent of the ABO groups can be prepared 1) if the plasma and the red cells are separated in a separator-type centrifuge, where all cells and plasmas are in touch with each other, 2) if the plasma is first removed from each blood bottle separately and some red cells are added to the pooled plasmas before final separation, or 3) if the plasma is taken from suitable calculated amounts of different bloods of the normal group titer $\alpha = {}^1/_{16}—{}^1/_{32}$, $\beta = {}^1/_8—{}^1/_{16}$ (6). In all three cases, the resulting serum contains some hemoglobin. If the blood groups are disregarded and the serum is prepared without mixing the cells and from such amounts of bloods of different blood groups as correspond to the normal blood group percentages in the population, the resulting serum has the titers $\alpha = {}^1/_4—{}^1/_2$, $\beta = {}^1/_2—{}^1/_1$ and is free of hemoglobin. The decrease of the titers when different cell-free plasmas are combined has been observed earlier (7) and might be explained by the occurrence of secretory antibodies in the plasma.

SUMMARY

Two methods are described for the preparation of normal human serum to be used as a plasma volume expander which stays in a good condition for 25 to 50 weeks of storage at room temperature.

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FROM THE DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF TURKU

CHLORPROPHENPYRIDAMINE AND PHENINDAMINE

EFFECT ON INCREASED OXYGEN CONSUMPTION PRODUCED BY ADRENALINE

by

O. J. KASSILA and M. K. PAASONEN

(Received for publication February 24, 1954)

In addition to their specific properties, the antihistamine substances have, among others, adrenergic or adrenolytic properties. The toxicity of adrenaline is increased by some antihistaminics and decreased by others (7). One of the typical antihistamine substances strengthening adrenaline toxicity is chlorphenpyridamine, whose ability to potentiate the effects of adrenaline on circulation was shown by Tislow *et al.* (10). The toxicity of adrenaline was lowered most clearly by phenindamine, whose antiadrenaline effect in blood pressure tests was demonstrated by Lehmann *et al.* (5).

According to Fabinyi-Szebehely and Szebehely (3), diphenhydramine potentiates the adrenaline-induced increase in oxygen consumption and rise in temperature. From their results, these authors concluded »that when the histamine sensitivity of the organism is lowered, the sensitivity to adrenaline is raised. This implies that the adrenaline-potentiating effect of diphenhydramine is connected with its antihistamine properties». However, this cannot be true, at least not always, because potent antihistaminics can be adrenolytic, as is mentioned above.

On the basis of the adrenolytic properties possessed by phenindamine, it is interesting to see its effect on adrenaline-increased metabolism, especially when compared with adrenergic chlorpro-

phenpyridamine. At the same time, this investigation complements our previous studies on the effects of antihistaminics on adrenaline responses in animals (7) and human beings (6).

METHODS

In the experiment white female rats, weighing 150 to 200 g, which were accustomed to these tests were used. They were given mixed feedings, except for the 24 hours before the test, when only casein and water were given. Basal metabolism was measured with the Richards and Collison apparatus (8), in which the test-animal is kept in an air-tight chamber in a water bath at 30° C. The carbon dioxide was combined into granulated soda lime.

Oxygen consumption was measured every 15 min. Before injections, it was measured three times, and the average of the last two values was calculated. This average is shown in Table 1 as normal oxygen consumption (A). After this, the drugs were administered and the oxygen consumption was again measured, beginning about 10 min. after the adrenaline injection for one hour at 15-min. intervals. The average of the two last measurements was noted when the increase of oxygen consumption after treatment was calculated (B). All values are averages obtained from experiments made with 10 rats in varying order. The adrenaline dose for each group of rats remained unchanged throughout the tests with each adrenaline dose. The time between two successive experiments was three days or more.

The following drugs were used: l-adrenaline hydrochloride (Adrenal, Orion OY., Helsinki, the dose expressed as base), chlorprophenpyridamine maleate (Chlor-trimeton, Schering Corp., Bloomfield, N. J.) and phenindamine hydrogen tartrate (Thephorin, F. Hoffman-La Roche & Co., Basle). All these drugs were diluted with saline solution and injected subcutaneously. The injected volume was 0.15 to 0.30 ml. When both antihistaminic and adrenaline were injected, the former was administered 10 to 15 min. before the latter.

RESULTS

In animals pretreated with 10.0 mg/kg of antihistaminic larger averages were obtained than with the 0.6 mg/kg dose of adrenaline alone, but the differences were far from significant (Table 1). This amount of chlorprophenpyridamine alone clearly raised the basal metabolism. In later experiments, the doses of antihistamine substances were smaller (0.5 to 2.0 mg/kg), especially that of chlorprophenpyridamine, in proportion to their different activity as histamine antagonists and therapeutic drugs.

It is curious that the 0.3 mg/kg dose of adrenaline produced

TABLE 1

EFFECT OF CHLORPROPHENPYRIDAMINE (C) AND PHENINDAMINE (P) ON THE OXYGEN CONSUMPTION INCREASED BY ADRENALINE IN RATS (MEAN ± S.D. OF THE MEAN)

A = Normal oxygen consumption in ml/100 g/15 min. B = per cent increase of oxygen consumption after treatment

Adrenaline Subcutaneously, Mg/Kg	Without Pretreatment		Pretreatment with Antihistaminics Subcutaneously 10 Min. before Adrenaline, Mg/Kg							
			C 0.5		P 2.0		C 10.0		P 10.0	
	A	B	A	B	A	B	A	B	A	B
Controls (without adrenaline)			37.2 ± 1.7	+ 2.9 ± 1.9			38.9 ± 1.4	+25.2 ± 4.7	40.1 ± 2.2	+ 7.2 ± 5.7
0.03	32.5 ± 1.2	+26.7 ± 4.2	33.9 ± 1.4	+37.2 ± 5.0	33.2 ± 1.3	+29.5 ± 3.8				
0.3	36.5 ± 1.6	+56.8 ± 4.6	39.7 ± 1.4	+44.9 ± 4.5	35.4 ± 1.6	+52.5 ± 5.7				
0.6	39.2 ± 1.7	+45.7 ± 9.2					43.4 ± 2.1	+54.3 ± 7.6	43.9 ± 2.2	+52.5 ± 8.5

reactions that seemed stronger than those brought about with the 0.6 mg/kg dose. In both cases it must be remembered that there is obviously a question of maximum effect or different sensitivity in laboratory animals. After chlorprophenpyridamine 0.5 mg/kg, the reaction did not seem as strong as in the control group with the 0.3 mg/kg adrenaline dose ($0.1 < P > 0.05$).

With 0.03 mg/kg of adrenaline, oxygen consumption was also increased, which shows that these rats were especially sensitive. The sensitivity was much greater than that reported by the Szebehelys (3), but their rats were anesthetized with urethane, which in our preliminary experiments seemed to block the adrenaline effect on metabolism. For example, in rabbits adrenaline injected subcutaneously in a dose of 0.03 mg/kg has slight effect on the blood sugar (11). The effect of this amount of adrenaline seemed to be strengthened by chlorprophenpyridamine in our tests, although not significantly ($0.2 < P > 0.1$).

In all test groups the effect of phenindamine seemed to go in the same direction as after chlorprophenpyridamine pretreatment. However, the difference in control groups was much smaller and never significant. (The injections of saline solution alone had no effects.)

DISCUSSION

In doses of 10 mg/kg, the antihistaminics themselves, especially chlorprophenpyridamine, increased the basal metabolism. It is known that in sufficiently high doses all antihistaminics are excitants to animals, but in smaller doses they are neither excitants nor sedatives. Phenindamine in contradistinction to other antihistaminics, which are sedatives, is merely an excitant to human beings (1, 2).

It is natural enough that the antihistaminics investigated had no clear effect on the ability of adrenaline to increase oxygen consumption. The adrenolytic and adrenomimetic properties of antihistaminics appear especially in toxicity and blood pressure tests, but not in metabolism. Although there is evidence that chlorprophenpyridamine in some animal tests potentiates adrenaline-induced hyperglycemia, while phenindamine reduces it (4, 9), we could not observe any of these reactions with rats (7). Phenindamine, on the contrary, increased the adrenaline responses in human

beings (6). In this study, phenindamine did not seem have any clear effect on the adrenaline responses, but chlorprophenpyridamine seemed to strengthen the effect of small adrenaline doses and to decrease the effect of large doses, although not very significantly. The fact that the standard deviation of control measurements is so small shows that the method is satisfactory, even though there was great variation in response.

SUMMARY

In rats, chlorprophenpyridamine in a dose of 0.5 mg/kg increased the oxygen consumption induced by adrenaline in a dose of 0.03 mg/kg, but decreased the effect of 0.3 mg/kg of adrenaline. After a large antihistamine dose (10 mg/kg), the response to 0.6 mg/kg of adrenaline was increased. Chlorprophenpyridamine alone in a dose of 10 mg/kg induced an increase in oxygen consumption. In all tests, the effects of chlorprophenpyridamine and phenindamine were similar, but the former was more effective.

We wish to thank the Schering Corp., Bloomfield, N. J., for chlorprophenpyridamine maleate (Chlor-trimeton); F. Hoffman-La Roche & Co., Basle, for phenindamine hydrogen tartrate (Thephorin), and Leiras, Manufacturing Chemists, Turku, for the test animals.

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FROM THE DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF HELSINKI

EFFECT OF CHLORPROPHENPYRIDAMINE ON PULMONARY EDEMA PRODUCED BY ADRENALINE AND CHLOROPICRIN IN RABBITS

by

M. K. PAASONEN and DANIEL ECKERT

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In the studies of the effect of some antihistaminics on adrenaline responses in animals, it was shown by one of us (M.K.P.) that there were two groups of antihistaminics, of which some increased and some decreased the adrenaline and noradrenaline toxicity (6). One of the most powerful substances of the former category was chlorprophenpyridamine, which in a dose of 10 mg/kg decreased the median lethal dose (LD_{50}) of adrenaline given subcutaneously in mice from about 8 to 3 mg/kg. It is recognized, on the basis of earlier experiments, especially those of Halpern and co-workers (litterature in 6) on promethazine, that antihistamine substances primarily prevent the pulmonary edema caused by adrenaline mainly through their ability to decrease the cellular permeability. According to Rothlin (8), vasopressin and natural ergot alkaloids which are vasoconstrictors, but not the hydrated vasodilating ergot alkaloids, blocked phosgene pulmonary edema. Hydergin did not prevent chloropicrin lung edema in rabbits (10). Antihistaminics are all strong vasoconstrictors, and chlorprophenpyridamine is one of the strongest, as is shown by their local effect upon the mammalian precapillary sphincters (2).

If the permeability-decreasing effect of antihistaminics is the principal reason for the capacity possessed by some of them to prevent adrenaline lung edema, this property might be revealed

also by chlorprophenpyridamine in experiments where its adrenaline-potentiating components are eliminated. Therefore the pulmonary edema in these experiments is produced by a cell-destroying substance, chloropicrin. In addition, the effect of chlorprophenpyridamine on adrenaline toxicity in rabbits is investigated, as was done in earlier tests with mice, rats and guinea-pigs.

METHODS AND RESULTS

Rabbits weighing 1,900 to 3,000 g were used in the tests. The effect of chlorprophenpyridamine on the pulmonary edema produced by intravenous injections of adrenaline and chloropicrin was examined. Injections were made into the marginal ear vein. In dissolving the adrenaline base (Rhône-Poulenc, Paris), a sufficient quantity of hydrochloric acid was used. The final volume of adrenaline solution injected was about 1 ml. Chloropicrin (Finnish Army Laboratory, Helsinki) was turned into a fine emulsion by dilution with water, containing polyethylene glycol, which formed 8 per cent of the final volume. The concentration used was 7.5×10^{-3}. Ten mg/kg of chlorprophenpyridamine maleate (Chlor-trimeton, Schering Corp., Bloomfield, N.J.) in a concentration of 0.5×10^{-2} was injected subcutaneously 30—45 min. before the administration of the substance under investigation.

A. Adrenaline. — After the adrenaline injection, the breathing of the rabbits in both groups became deep and quick after the short apnea. Death usually followed vigorous tonic-clonic convulsions and the issuance of bloody froth from the nostrils. From Table 1 we can see that the lethal dose of adrenaline in the chlorprophenpyridamine-pretreated rabbits was smaller than that in the controls. On the other hand, it must be noticed that according to the tests the survival time after pretreatment seems longer. At the autopsies severe pulmonary edema was regularly found. In both lungs there were large hemorrhagic areas, from which bloody fluid issued on operation. It was impossible to note any difference between the average per cent lung weights of both groups.

B. Chloropicrin. — After the chloropicrin injections, the animals often squealed piercingly for a little while and were very restless. Later on, their physical reactions were very similar to those described above, and death followed severe convulsions. Chlorprophenpyridamine-pretreated animals tended to withstand chloropicrin more easily. From the average lung weights, nothing can be deduced

TABLE 1

EFFECTS OF CHLORPROPHENPYRIDAMINE ON THE LETHAL DOSE OF ADRENALINE AND CHLOROPICRIN IN RABBITS

A = Controls, B = Pretreated with 10 mg/kg of Chlorprophenpyridamine 30—45 Min. before the Injection of Adrenaline or Chloropicrin

	Mortality Ratio[1]		Average Lung Weight %[2]		Survival Time, Min.[3]	
	A	B	A	B	A	B
Adrenaline, mg/kg intravenously						
0.2	0/3	1/3		1.2		12
0.4	0/3	3/3		1.1		8, 14, 20
0.6	0/4	4/4		1.1		11, 14,[4],[4]
1.0	5/7	2/2	1.1	1.2	4, 4, 5, 5,[4]	[4], [4]
Chloropicrin, mg/kg intravenously						
15	0/3	0/3				
20	1/5	0/5	1.0		96	
30	3/6	1/6	1.2 (1.1)	1.3 (1.0)	26, 40, 51	46
40	7/7	4/7	1.4	1.4 (1.1)	20, 25, 30, 36, 42, 50, 61	30, 40, 120, 190

[1] Number killed/number in groups.

[2] Includes those animals which succumbed after injection. Values in parentheses are for survived animals killed with a blom on the head four hours after chloropicrin injection.

[3] Excluding those surviving indefinitely.

[4] Survival time, 2 to 10 hours.

but that the two animals with the longest survival time belonged to the pretreated group. On autopsy severe hemorrhagic pulmonary edema was observed, but there was not so much congestion in the heart as was the case with adrenaline.

DISCUSSION

It is interesting to note that in the adrenaline toxicity tests the survival time of animals pretreated with chlorprophenpyridamine was longer than that of the controls, although the mortality rate was higher in the former group. It has been ascertained that antihistaminics are a stronger preventive of adrenaline-induced premature ventricular contractions than quinidine is (1, 9, 11). In our blood pressure experiments, this characteristic was shown to be

common to chlorprophenpyridamine also (6). If the function of the heart is important in adrenaline lung edema, one can think that antihistaminics lengthen the lifetime by preventing adrenaline's unfavorable effect on the heart. Anyway, this would not prevent the development of the final fatal lung edema, for which even much smaller doses than those in the control group would be adequate. Nickerson *et al.* (5) administered adrenaline intraperitoneally in rats and observed that pulmonary edema is perceptible only when the animals are kept alive sufficiently long by artificial respiration or systemic arterial pressure stabilization.

In the chloropicrin tests, the result was different from that in the adrenaline tests. According to Table 1, the lethal dose after chlorprophenpyridamine pretreatment was larger, although the difference was not great. In the group that had received the antihistamine substance, the longest lifetimes were found. In both groups the average lung weights for the control and the pretreated animals were quite comparable. These results resemble those of Halpern *et al.* (3) in similar tests with promethazine, although the LD of chloropicrin in our experiment was considerably greater.

This study indicates that chlorprophenpyridamine is similar to other antihistaminics in preventing the pulmonary edema produced by irritant agents, such as chloropicrin. Perhaps this is also true of both diphenhydramine and mepyramine, although they like chlorprophenpyridamine increased the toxicity of adrenaline (6).

It must be remembered that antihistaminics fail to affect the pulmonary edema produced by bilateral cervical vagotomy (6, 7) and ammonium chloride (6, 12), although in the case of vagotomy at least one factor is believed to be the increased permeability. The role of histamine in various types of lung edema may be different, and, therefore, the results in various experiments differ from each other. Chlorprophenpyridamine, for example, did not have any effect on capillary permeability, when the extravasation of intravenously injected trypan blue was examined around the site of ntradermal injection of trypsine or pantocaine (6). This indicates that the permeability-decreasing effect of antihistaminics depends only on their histamine antagonistic properties.

This experiment testifies to chlorprophenpyridamine's capacity for intensifying the lung edema induced by adrenaline. In spite of that, its prventive effect on the development of the lung edema is

maintained, if the increase in permeability has been brought about in such a way that adrenomimetic properties of the drug do not appear.

SUMMARY

Chlorprophenpyridamine (10 mg/kg) injected subcutaneously in rabbits increased the toxicity of intravenously injected adrenaline. The survival time after pretreatment, however, was longer, indicating that the mechanism of pulmonary edema, which was believed to be the principal reason for death was different from that in the control group. The same pretreatment decreased the toxicity of intravenously injected chloropicrin to some degree.

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FROM THE DEPARTMENT OF PHYSIOLOGY, INSTITUTE OF OCCUPATIONAL HEALTH, HELSINKI

REGULATION OF TIDAL AIR

IN RELATION TO VENTILATORY EQUIVALENT AND OXYGEN REMOVAL IN PULMONARY INSUFFICIENCY DURING EXERCISE

by

TEUVO K. I. LARMI

(Received for publication February 25, 1954)

Christensen (5) pointed out several years ago that, during and with increasing loads of exercise, the rise in ventilation is caused by the increased tidal air and respiratory frequency. On exercise with increasing loads, the rise in ventilation is, however, caused mainly by the increased frequency. He pointed out, further, that tidal air is adjusted optimally from the point of view of respiratory work. Later, this fact was established with certainty, but it was noted that each alveolar ventilation has optimal respiratory frequency when the work used for breathing is smallest (18). Christie (6) observed that the respiratory rhythmicity is optimal in its relation to the respiratory work in patients with congestive heart failure and with pulmonary emphysema. In his opinion, the regulation of this frequency is important from the point of view of dyspnoea.

Similar findings were observed in subjects with various types of pulmonary insufficiency (12). However, it was established that during graduated exercise the correlation between the tidal air and end-tidal air pO_2 and pCO_2 changes with increasing loads of exercise. It was noted that, at rest or during lighter exercise, pO_2 rises in relation to the increase of the tidal air. On the contrary, on exercise with increasing loads the decrease of the tidal air is connected with the increase of end-tidal air pO_2. As this type of finding occurs mostly in subjects with left heart failure, the conclusion may be drawn that

the regulation of the tidal air during exercise is possibly connected with alveolar ventilation-perfusion ratio. The basis for this hypothesis is, however, formed merely by the findings concerning the tidal air and the partial pressures of end-tidal air pO_2 and pCO_2. The present work endeavours to examine, with similar subjects, the relation between the tidal air and ventilatory equivalent and between the tidal air and oxygen removal.

METHODS

The subjects and the methods applied are described in more detail in another connexion (12). The series consisted of 104 patients, divided into the following groups: 1) control group, with neither cardiac nor pulmonary diseases, 2) subjects with pulmonary disease without emphysema, 3) subjects with emphysema, 4) subjects with cor pulmonale, 5) subjects with left heart failure and 6) subjects with left heart failure and complicating pulmonary disease.

The ventilation was examined with a gasometer (16), and the respiratory frequency was recorded through the rotation of the drum. The average tidal air of the subjects was determined from the ratio of minute volume of ventilation and respiratory frequency, and the values were corrected according to BTPS. The oxygen consumption was determined from gas samples with the micromethod of Scholander (20); the values obtained were corrected according to STPD. The ventilatory equivalent and oxygen removal were calculated on the basis of the quantities mentioned above, according to the usual criteria (2, 11). Short-time graduated exercise with the bicycle ergometer was used (12). The covariance analysis was used for mathematical method.

RESULTS

Correlation between the Tidal Air and Ventilatory Equivalent. — The tidal air and ventilatory equivalent showed strong linear correlation at rest and with a load of 300 kgm/min. ($p < 0.1$ per cent) and almost significant linear correlation with a load of 700 kgm/min. ($p = 5$ per cent) for the entire series. On the other hand, with loads of 500, 600 and 900 kgm/min., the correlation coefficient did not differ significantly from zero. In examinations with the aid of covariance analysis, the result was negative with loads of 300 and 900 kgm/min., if the deviations from linearity could be explained by original variation, *i.e.*, on these exercises the correlation of the entire series cannot be presented with the same straight line ($p < 0.1$ per cent and $p = 4.2$ per cent, respectively). This finding can be explained

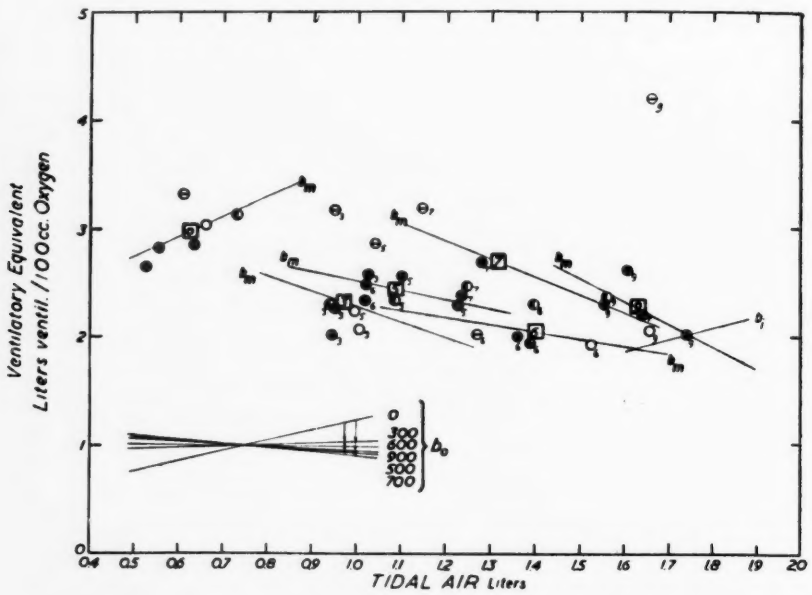

Fig. 1. — Correlation between tidal air and ventilatory equivalent at rest and on graduated exercise on an average in the various groups. The regression of means (b_m) at rest and on various exercises is shown (note the turning of the regression line with increasing loads). At the lower left corner, the lines of average regression in the entire series (b_0) at rest and on various exercises are presented, the arrows showing the turn with increasing loads of exercise (note the differences: b_0 = physiological effect and b_m = patho-physiological effect). The regression within Group 5 with a load of 900 kgm/min. is illustrated (b_i). Its course is positive, the course of the regression within the other groups being negative.

Symbols: Means of the various groups: ○ = Group 1, ◎ = Group 2, ◐ = Group 3, ⊖ = Group 4, ⊕ = Group 5, ● = Group 6. The means, without index numbers are values at rest; the index number shows the load in kgm/min. × 100 presented by each mean (*e.g.*, $\ominus_3$ = Group 4 with a load of 300 kgm/min.). The means in the entire series = □. The index number inside the symbol indicates the equal load as the indexes for the various groups (*e.g.*, [0] = the mean of the entire series at rest, etc.).

by the fact that the deviation of the means of various groups from the regression line of the means (b_m) was of statistical significance ($p < 0.1$ per cent and $p = 3.1$ per cent, respectively). This finding is illustrated in Fig. 1. It can be seen that with loads of 300 and 900 kgm/min. both Group 4 and Group 6 deviate upward along the y-axis, while with a load of 300 kgm/min. Group 1 and Group 5 and with a load of 900 kgm/min. only Group 1 deviate downward along the

y-axis. The finding shows that the ventilatory equivalent is in the former groups relatively higher, on an average, and in the latter groups relatively lower than the equal tidal air had provided, compared with the other subject groups. Though the results on other exercises had no statistical significance, a similar tendency was noted in all of them. It is worth emphasizing that Groups 5 and 6 (with left heart failure) differed from each other in this respect.

While moving from rest to exercise, as well as with increasing loads of exercise, it was noted that the mean regression of the entire series (b_o) showed highly significant turning as a result of exercise ($p < 0.1$ per cent). This turning is also presented in Fig. 1. The values of the corresponding coefficients of regression are to be seen in Table 1. It can be noted that the regression was positive at rest, *i.e.*, the ventilatory equivalent increased in the entire series with the increase of the tidal air, and *vice versa*. When moving to exercise, the course of the regression was already slightly negative with a load of 300 kgm/min. On exercise with prolonged effort, it proved to be increasingly negative, the turning being stronger with loads of 500 and 700 kgm/min. than with loads of 600 and 900 kgm/min. The subjects of the former group were patients with inferior endurance for exercise and with less ability to increase ventilation than the others (12). Thus, exercises with increasing loads changed the correlation between the tidal air and ventilatory equivalent: The larger the tidal air, the smaller the ventilatory equivalent, and *vice versa*.

When comparing the turning of the regression of the means (b_m) and the turning of the regression of the entire series (b_o) due to exercise (Fig. 1, Table 1), it was noted that the regression of the means (patho-physiological effect) turned more sharply than the average regression of the entire series (physiological effect). Accordingly, the physiological effect in some ways resisted the sharpness of turning. However, the difference was of no significance due to the great scatter.

A similar statement can be made for the difference of regressions (b_i) in various groups both at rest and during exercise. In that case, the differences between the regression of means (b_m) and the average regression within the group (b_a) were not significant. However, it was noted in Group 5 that on lighter exercise, with the increase of the tidal air, the ventilatory equivalent, in

TABLE 1

NUMBER OF SUBJECTS IN ENTIRE SERIES AT VARIOUS EXERCISES AND VARIOUS COEFFICIENTS OF REGRESSION IN CORRELATION INVESTIGATION OF TIDAL AIR AND VENTILATORY EQUIVALENT, AND IN CORRELATION INVESTIGATION OF TIDAL AIR AND OXYGEN REMOVAL

Number fo Subjects	Exercise, Kgm/Min.	Correlation between Tidal Air (Liters) and Ventilatory Equivalent (Liter Ventil./ 100 Cc 0_2)			Correlation between Tidal Air (Liters) and Oxygen Removal (Cc 0_2/Liter Ventil.)			Remarks
		b_a	b_m	b_o	b_a	b_m	b_o	
104	0	+0.775	+1.78	+0.82	—9.32	—13.84	—9.53	No significant difference between b_a and b_m
104	300	—0.039	—1.42	—0.055	+2.55	+ 1.76	+2.54	Linearity of group means poorer than linearity in groups ($p<0.1$ per cent)
51	500	—0.288	—0.847	—0.320	+5.27	+13.17	+5.72	Coefficient of correlation does not differ significantly from zero
53	600	+0.026	—0.710	—0.039	—2.36	+18.55	+0.52	Coefficient of correlation does not differ significantly from zero
29	700	—0.258	—1.58	—0.544	+6.54	+20.90	+9.65	Coefficient of correlation differs from zero almost significantly
54	900	—0.227	—1.88	—0.279	+4.28	+36.00	+5.28	Group 5 inclines to differ from the others

b_a = average regression within the group
b_m = regression of the means
b_o = coefficient of regression in the entire series

general, decreased less than the other on an average. With loads of 600 and 900 kgm/min. in Group 5, the regression within the group (b_i) was positive, especially with a load of 900 kgm/min., while for all the other groups the regression proved to be negative (Fig. 1). Group 6 (subjects with left heart failure and complicating pulmonary disease) proved to be strongly negative during all the experiments. Regularity was noted also in Group 3 (subjects with emphysema), in which the regression was positive up to the load of 600 kgm/min., turning thereafter to negative.

Correlation between Tidal Air and Oxygen Removal. — The investigations concerning the correlation between the tidal air and the oxygen removal approximately agreed with the results reported

above. The statistics, however, were less obvious. The correlation proved to be linear at rest and with loads of 300 and 700 kgm/min. ($p = 0.2$ per cent, $p = 0.4$ per cent, and $p = 5$ per cent, respectively) in the entire series. The variations from linearity were not explained by the original variation with a load of 300 kgm/min., due to the fact that the means of various groups deviated notably from the regression line of means ($p = 0.1$ per cent). Group 4 and Group 6 had lower oxygen removal than the other groups at equal tidal air. On the other hand, the oxygen removal was respectively greater in Group 5.

On exercise with increasing loads, the mean regression of the entire series (b_0) turned ($p = 0.2$ per cent). The course of the regressions was at rest and during exercise naturally opposite to that in the correlation between the tidal air and ventilatory equivalent (Table 1). The other results obtained from correlation investigations of the tidal air and the oxygen removal agreed with those of the tidal air and the ventilatory equivalent.

DISCUSSION

Christie (6) pointed out that orthopnoea and dyspnoea in patients with heart failure may be due to increased respiratory work caused by pulmonary congestion. In his opinion, typical rapid and shallow resspiration in patients with congestive heart failure is due to the fact that the rhythmicity is arranged most economically in terms of respiratory work. He pointed out, further, that the resistance caused by the viscoresistant properties of the lungs (14) is, especially in voluntary hyperventilation, greater than on exercise in patients with emphysema. It is obvious that the physiological regulation of the tidal air whatever its aims may be is based mainly on the functions of the pressure receptors in the lung parenchyma (13, 19). It seems likely that among the additional forces activating the pressure receptors is the increased pressure of the pulmonary artery. However, with this increased pressure there occur distinct functional differences in the patients with left heart failure and in the patients with primary pulmonary disease (4, 9, 15).

Left heart failure is caused mainly by the stress on the left ventricle. Increased pulmonary artery pressure may be caused by the changes in composition of the respiratory gases in blood and in alveolar air (1, 7, 17). Accordingly, it may be assumed that, in pati-

ents with pulmonary disease, the decreased alveolar oxygen pressure or the increased carbon dioxide pressure would be among the forces causing acceleration of respiration. This is probably not the case in left heart failure. It may be possible, then, that the increase of the end-tidal air pO_2, owing to left heart failure, with shallow respiration (12) should be aimed also at functions other than respiratory work. It seems likely, on the basis of the results of the present study, that this might be the case in Group 5, which consisted of patients with left heart failure. Similar findings could not be stated for Group 6, consisting of patients with left heart failure complicated with pulmonary disease. Typical of both groups, on the basis of results reported in another connexion (12), was the fact that on exercise with increasing loads during the decrease of the tidal air the end-tidal air pCO_2 also decreased and pO_2 increased. Thus, the conclusion can be drawn that in patients with left heart failure the decrease of the tidal air on exercise is not necessarily connected with the increase of ventilatory equivalent or the decrease of oxygen removal. Rather the case may be the opposite. On the other hand, if left heart failure is complicated with pulmonary disease, the situation is, again, different. Because the increased functional residual air was observed mostly in the groups with pulmonary disease and because the effectiveness of alveolar ventilation seemed to be reduced on exercise (12), the differences may be due to these facts. Emphysema, especially, seems to play an important role in this respect (3). In general, the regulation of the respiration, its rhythmicity and total ventilation in pulmonary insufficiency form a highly complicated problem (8, 10, 21). The results reported in the present study cannot be taken as a full evaluation of evidence, and the problem needs further investigation.

SUMMARY

On the basis of the methods reported in another connexion (12), 104 subjects presenting various types of pulmonary insufficiency were investigated concerning the correlation between the tidal air and ventilatory equivalent and the correlation between the tidal air and oxygen removal on short-time graduated exercise. The author states his working hypothesis as follows: acceleration of respiration due to left heart failure may be not only optimal as to the respira-

tory work, but also favourable as to the ventilatory equivalent and oxygen removal.

The results showed that on exercise with increasing loads the decrease of the tidal air in patients with left heart failure may be connected with the increase of oxygen removal and the decrease of ventilatory equivalent. The case was the opposite in the other groups with pulmonary insufficiency and in the control group. In the group with left heart failure and complicating pulmonary disease, the results disagreed markedly with findings for the first-mentioned group. A short discussion on the results obtained is given on the basis of earlier investigations.

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FROM THE STATE SERUM INSTITUTE, THE VIRUS LABORATORY, UNIVERSITY OF HELSINKI, AND THE OFFICE OF THE SURGEON GENERAL, FINNISH DEFENCE FORCES

STUDIES OF VACCINATION WITH FORMOLISED MUMPS VIRUS VACCINE IN THE FINNISH DEFENCE FORCES

by

KARI PENTTINEN[1], PEKKA SOMER, ERKKI KLEMOLA and ERKKI KIVALO

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Several attempts have been made to prevent mumps by different types of mumps virus vaccines (17, 2, 16, 3, 5, 6, 9). Both attenuated active virus (4, 9) and inactivated mumps virus vaccines have been used. The most extensive studies were carried out by Habel (5) and Henle and co-workers (8, 7, 6, 9, 1). The former vaccinated 1,344 humans beings judged susceptible and the latter 250. From the results of these studies and the others mentioned above, it can be concluded that the protective effect of the various mumps virus vaccines is unquestionable. The degree of prevention, however, varies with the amounts of virus injected and the interval between vaccination and infection.

The results of the vaccination studies referred to above led to the thought that with a mumps virus vaccine protection against mumps could be achieved in army units in Finland, populations in which mumps and its complications occur inevitably. Mumps, especially mumps orchitis, is a constant problem in the Finnish Defence Forces (10). The yearly incidence in 1946 to 1953 varied between 445 and 1,023 cases (average, 681 cases). With the total strength of the Defence Forces at about 30,000 in all ranks, 1.5 to 3.5

[1] Aided by a grant from the Sigrid Jusélius Foundation.

per cent of the new recruits contract mumps each year. If the percentage of orchitis is taken as 20 of all mumps cases, the average number of orchitis cases each year is of the order of 140. Since 10 to 20 per cent of the cases of mumps orchitis are bilateral and about 50 per cent of the patients remain sterile according to Lambert's very comprehensive study (11), mumps probably results in an average of ca. 10 cases of sterility a year in the Finnish Defence Forces. Taking into consideration, further, that the period of service of a national serviceman in the army is usually only eight months, during which time vaccination might have enough effect, the authors started a vaccination experiment with formolised mumps virus preparations. The results of this vaccination experiment are reported in the following.

METHODS

Mumps Virus Vaccine. — All the different lots of vaccine were made with the egg-adapted Enders strain of mumps virus (courtesy of H. R. Morgan, 1949). The number of egg passages before arrival in our laboratory is unknown. The vaccines were made from 9—16 egg passages in our laboratory. Lots of eggs incubated at 38° C for 7—8 days were infected intra-allantoically (0.2 ml) with 10^{-3} dilution (broth) of infected allantoic fluid. After 5 to 6 days' incubation at + 36° C, the living eggs were chilled and the allantoic fluids collected. Only occasionally were dead eggs found. The pooled allantoic fluids were centrifuged for 10 min. at 1,500 rpm, and 0.3 per cent of formalin was added to the supernatant. After 48 hours at + 4° C, the fluids were centrifuged at 15,000 rpm for 45 min. in a Spinco Ultracentrifuge. The supernatant was discarded, and the sediment was suspended in buffered saline solution containing 0.1 per cent phenol and 0.01 per cent Merthiolate. The concentrated mumps virus vaccine was again centrifuged for 10 min. at 1,500 rpm, and after suitable dilution placed in ampoules and stored at + 4° C. Controls were made from each lot for sterility, viability and virus strain.

For the vaccination of the 1,830 men of our series, allantoic fluids were collected from 1,275 eggs. The haemagglutinating titre of allantoic fluid pools varied from 1: 512 to 1: 2048. The 50 per cent infectivity endpoint was of the order 10^{-8}—10^{-9}. The total amount of allantoic fluid harvested was 4,943 ml, an average of 3.9 ml per egg. In this vaccination study, each vaccinated man received 0.7 egg dose of mumps virus, if we assume that the amounts of virus lost were small.

A total of nine lots of vaccine was produced in all. Lots No. 4 and 5 were combined, and the other lots were placed in ampoules separately. The concentrated vaccines were diluted to haemagglutinating titre 1: 256 before ampouling. When, however, the vaccines were tested again before

delivery, it was observed that the haemagglutinating titre had dropped considerably. With sonic treatment of the samples of the vaccines, it was, however, possible to show that the drop in titre was largely misleading (14).

In comparing influenza and mumps virus vaccine production, it might be said that, generally, the haemagglutinating titre of mumps virus seems to be much more sensitive during the different phases of production. This does not necessarily mean that the immunising power is lost with decreasing haemagglutinating titre, although the lost haemagglutinins could not be detected with sonic treatment, for example.

Laboratory Tests. — The techniques in haemagglutination and haemagglutination inhibition tests, as well as in complement fixation, were described in an earlier publication (13). Some minor modifications should be noted: In haemagglutination inhibition and haemagglutination titrations, plastic plates were used (15). The results of the complement fixation test were read by eye with the aid of 50 per cent haemolysis controls. Before the reading, the tubes were kept overnight at + 4° C. A tube showing a haemolysis equal to or less than the 50 per cent control was considered positive.

The Vaccination. — Taking into consideration the fact that the determination of susceptibility with the skin test or serologic tests is time consuming and somewhat uncertain, it was decided to limit the number of men to be vaccinated merely by questioning them regarding possible earlier mumps infection. Of 5,654 men enrolled at the beginning of November, 1952, 1,629 (28.8 per cent) told when questioned of having had the disease and 4,025 (71.2 per cent) maintained the contrary. This large number of men with a negative history of mumps was, from earlier studies, somewhat unexpected (13). Of the men with a negative history of mumps, 297 were excluded for various reasons, and of the remainder, 1,830 received mumps virus vaccine, while 1,898 (99 in the control group received a 0.1 ml dose of vaccine) served as controls. The division into two groups was carried out according to alphabetical order, every other man being vaccinated. This procedure was adopted in all the 22 army units involved in the study.

The vaccine was administered in two doses. The first 0.1 ml subcutaneous dose was given to test for possible sensitivity to egg protein; the second subcutaneous dose, 0.5 ml, was considered as the immunising dose proper. The second dose was given one week after the first.

A 10 per cent sample of paired sera was taken from the vaccinated men for serologic studies. The first blood sample was collected before the 0.1 ml vaccine injection, and the second sample three to five weeks after the first. Altogether, 201 paired sera were collected.

All records concerning mumps contracted by the men of the three groups (those with positive history, non-vaccinated men with negative history and vaccinated men with negative history) were collected and analysed. All men who acquired the disease were hospitalised.

RESULTS

Table 1 shows that, of 1,830 vaccinated men with a negative history of mumps, 46 or 2.5 per cent contracted the diseases; of 1,898 non-vaccinated controls, 86 or 4.5 per cent contracted mumps. The difference is not great, but it is statistically significant. Accordingly, it was possible to decrease the incidence of mumps by means of the vaccine and method of vaccination used in the present study. It would be well to mention in this connexion that, of the 1,629 men who stated they had had mumps previously, 9 or 0.55 per cent contracted the disease again during their army service.

TABLE 1

INCIDENCE OF MUMPS AND MUMPS ORCHITIS IN VACCINATED AND NON-VACCINATED GROUPS WITH A NEGATIVE HISTORY OF MUMPS

	Vaccinated, 1,830 Men		Non-vaccinated, 1,898 Men	
	Number	%	Number	%
Cases of mumps (132)	46	2.5	86	4.5
Cases of mumps orchitis (19) ..	1	0.05	18	0.95

Although vaccination had no startling effect on the incidence of mumps, it clearly influenced the nature of the disease, at least in regards to the frequency of orchitis complications. It is clear from Table 1 that of the 46 men who contracted mumps despite the vaccination only 1 (2 per cent) had orchitis. Of the 86 non-vaccinated men with mumps, 18 had orchitis (23 per cent). The figure corresponds on the whole with the frequency of orchitis reported in the literature. Of the 18 men with orchitis in non-vaccinated group 5 had it bilaterally. The only orchitis in the vaccinated group was bilateral and occurred 7 weeks after vaccination.

The possible moderating effect of vaccination on the course of mumps may also be seen from Table 2, where there are shown the mean maximum body temperature in the men with mumps, the total number of fever days, the duration of fever, as well as the number of men with secondary fevers.

According to Table 2, it is possible, although statistically difficult to maintain, that the vaccination saved a considerable number of fever days, shortened the duration of the fever and smoothed out the fever peaks somewhat. Clinical results pointing in the same

TABLE 2

MAXIMUM BODY TEMPERATURE, NUMBER OF FEVER DAYS, DURATION OF FEVER AND NUMBER OF MEN WITH SECONDARY FEVERS IN THE VACCINATED AND NON-VACCINATED GROUPS

	Vaccinated Group	Non-vaccinated Group
Cases of mumps	46	86
Maximum body temperature (arithmetic mean)	37.86°C	38.32°C
Sum total of fever days	146	360
Duration of fever (arithmetic mean)	3.2 days	4.2 days
Number of cases with secondary fevers	9	22

direction were obtained by Habel (5). As far as secondary fevers are concerned, it is difficult to say what proportion were brought on by general infections other than mumps in the military hospital.

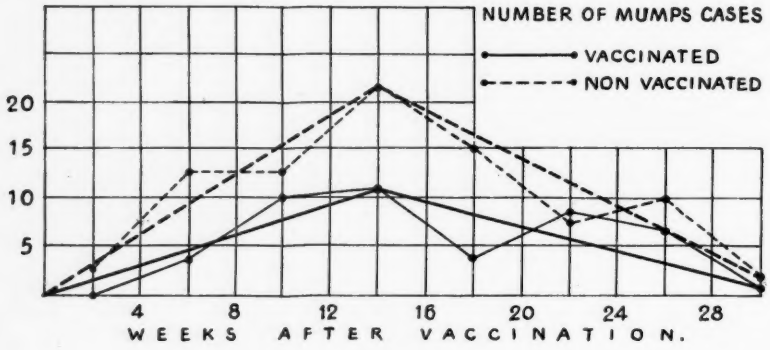

Fig. 1.

Fig. 1 presents the monthly numbers of mumps cases among the vaccinated and non-vaccinated groups. The number of cases in both groups reached its peak 12 to 16 weeks after vaccination (14 to 18 weeks after enrolment) and decreased again toward the end of the period of military service. The vaccinated group showed a smaller number of mumps cases for approximately a half-year after vaccination. The corrected curves may give a better picture of the behaviour of the different groups.

Not a single vaccination reaction of any type has been reported by the men vaccinated.

Serologic Investigations. — As mentioned earlier, 201 paired sera were collected from the vaccinated men. This represented a little more than a 10 per cent sample of all the vaccinated. When investigating the serum pairs by complement fixation and haemagglutination inhibition techniques, it was found that with the former technique the minimum geometric mean increase in antibody titre was 3.1-fold (from 0 to 32-fold), while with the latter the increase was 1.6-fold (from 0 to 16-fold). Due to its higher sensitivity in these series, only results with the complement fixation technique are referred to in the following. In this report, the main attention is paid to the behaviour of the group showing no antibodies in pre-vaccination sera as compared with that of the group with antibodies in pre-vaccination sera.

On study of the results of complement fixation tests, it was found that 56 sera (27.9 per cent) collected before vaccination showed no antibodies when the first tested dilution was 1: 4. Accordingly, at least 72.1 per cent of the men with a negative history of mumps had had some contact with mumps virus.

From the results, it seems probable that in most of the vaccinated men the vaccine administered served as a booster dose. If it is assumed that the group showing no antibodies before vaccination comprises those with no experience of mumps virus, the antibody response to vaccination should be less in this group than in the one with measurable antibodies in the pre-vaccination sera. The difference between the geometric mean antibody increases in both groups is presented in Table 3. The table also gives special consideration to non-reactors after vaccination.

In Table 3, it should be noted that the figures, although presented

TABLE 3

GEOMETRIC MEAN ANTIBODY INCREASES IN THE GROUPS WITH »NO ANTIBODIES BEFORE VACCINATION» AND WITH »MEASURABLE ANTIBODIES BEFORE VACCINATION»

	Geometric Mean Antibody Increases	
56 paired sera with pre-vaccination titre 1:<4	2.8-fold (minimum increase)	21 non-reactors excluded 5.1-fold
145 paired sera with pre-vaccination titre 1: $\geqq 4$	3.3-fold	20 non-reactors excluded 3.9-fold

in the same table, are not comparable with each other. Because of unknown changes in antibody titre below the lowest level investigated (1: 4), all conclusions are uncertain. Possibly the group with »no antibodies before vaccination» contained many men with experience of mumps. The results of neutralisation tests will be published later on. It can also be seen from Table 3 that 21 (38 per cent) of the men with a prevaccination titre 1: <4 did not react with antibodies to the vaccine stimulus; the corresponding figure in the other group was 20 (14 per cent).

Finally, it might be mentioned that paired sera were collected from five of the 46 vaccinated men who contracted mumps. All five belonged to the group with pre-vaccination titre 1: <4. In four of them, no measurable antibodies were observed after vaccination; the remaining man had a $\pm$ postvaccination titre in the dilution 1: 4.

DISCUSSION

The vaccination study reported on here has some special features. The vaccination was carried out in a population in which the great majority of the vaccinated men had had experience of the virus in question. Secondly, the dose of vaccine administered was small compared with *e.g.*, the dose administered by Henle and co-workers (10 egg doses) (6).

The estimation of susceptibility to mumps virus infection seems to be a difficult task (8). The history of past mumps can give only rough guidance in selecting the more susceptible part of the population to be vaccinated. With this means, however, it was possible to divide the population into two groups with a very different incidence of mumps. Among 1,629 men with past mumps infection, 9 (0.55 per cent) contracted the disease, and among 1,898 non-vaccinated men with a negative history of mumps, 86 (4.5 per cent) acquired the disease. Whether these 9 cases are due to incorrect information concerning past mumps or are real reinfections, it is impossible to say. The serologic study showed that the majority (72 per cent) of men vaccinated according to the negative history of mumps had had experience of mumps virus. The behaviour of the group with negative pre-vaccination sera (28 per cent) suggested that this group might also contain men with experience of mumps virus.

After all, the vaccination was carried out in a population in which, in most cases, the vaccine administered represented a booster dose. This can, however, be considered justifiable when only few complications from the vaccination are expected, as was the case in this experiment.

According to Lambert (11), the risk for persons with a negative mumps history to contract the disease is eight times that facing persons with a positive history. Our figures give the same relationship.

The clinical results also show that with small doses of vaccine relatively good results can be expected. It can be thought that the prevention of mumps orchitis is due to a low-grade immunity that increases during the primary phase to a degree inhibiting the multiplication of mumps virus on a scale which could cause serious effects in susceptible cells (18). The same protection from smalldose vaccination may be expected in other virus diseases with a primary and secondary phase and in which the complications are due to the destruction of susceptible cells. It is possible that the first small dose in this vaccination experiment had an favorable effect on the result.

As to the small amount of vaccine administered (ca. 0.7 egg dose), it can be thought that the drop in haemagglutinating titre before vaccination has a favourable effect on the immunising power. Clumping of virus particles or the masking of viruses in some other way may have the same effect as adjuvants: slower resorption.

SUMMARY

A vaccination experiment against mumps in the Finnish Defence Forces is described. The vaccine dose was of the order of 0.7 egg dose. The vaccination and control groups were selected according to the negative history of mumps. The groups of 1,830 vaccinated and 1,899 non-vaccinated men were compared. In addition, observations were made on a group of 1,629 men with a positive mumps history.

The results show that the incidence of mumps in the non-vaccinated group was 4.5 per cent (86 cases) and in vaccinated group 2.5 per cent (46 cases). The incidence of mumps orchitis in the non-vaccinated group was 0.95 per cent (18 cases) and in vaccinated group 0.05 per cent (1 case). The vaccination seems to have a favourable effect on the maximum temperature and duration of

fever. In the group of men with a positive history of mumps, the incidence of mumps was 0.55 per cent (9 cases).

The complement fixation tests with 201 paired sera showed that at least 72 per cent of the men with a negative history of mumps had had experience of mumps virus. Thirty-eight per cent of the men with a pre-vaccination titre 1: <4 did not react with complement fixation antibodies to the vaccine stimulus; the corresponding figure in the group showing antibodies before vaccination was 14 per cent.

The results are discussed.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

ANTIBIOTICS AND EXPERIMENTAL TOXOPLASMOSIS

II

ERYTHROMYCIN, MAGNAMYCIN, NEOMYCIN AND TEBEZON [1] IN EXPERIMENTAL TOXOPLASMOSIS [2]

by

PAUL GRÖNROOS [3]

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Of the commercially available antibiotics, aureomycin has been shown to have some effect on acute experimental toxoplasmosis (2, 3, 5). Terramycin has no or only slight effect (1, 2, 3), while bacitracin and polymyxin are without effect (3).

Grassi conducted experiments with various thiosemicarbazones in experimental toxoplasmosis in white mice. The drugs were administered intramuscularly in doses of 1 mg and were found to be without effect (2).

I have found no references in the literature concerning the effect of Neomycin, magnamycin and erythromycin.

Technique. — Ten-day old embryonated hen's eggs were used in the experiments. The technique was identical with that reported earlier (3). In these experiments a sulfa preparation (Elkosin, Ciba) was also used in a concentration of 40 mg per 0.2 ml dose as a control agent.

Magnamycin was used in a concentration of 5 mg/0.2 ml. The median lethal dose (LD_{50}) for magnamycin in hen's eggs was found to be 6 to

[1] p-Acetylaminobenzaldehydethiosemicarbazone was kindly placed at my disposal by OY. Medica AB.

[2] This investigation was aided by a grant from Svenska Vetenskapliga Centralrådet.

[3] With the technical assistance of Mrs. Pirkko Koivunen.

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7 mg/0.2 ml by the yolk-sac route of inoculation (7). Erythromycin was given as a saturated solution in a buffer of *p*H 7.4. The solution contained about 2 mg/ml of erythromycin (4). Neomycin was used in a concentration of 1 mg/0.2 ml, which is two to three times as high as that used by Waksman in some experiments (6). Tebezon was given in a concentration of 5 mg/0.2 ml.

TABLE 1

CHEMOTHERAPEUTIC EFFECT OF THE DRUGS

	No. of Eggs	Days after Inoculation									
		A								B	C
		2	4	5	6	7	8	9	10		
Toxoplasma 1/10 in buffer	14		1		6	7					
» » and sulfa	14					2			2	9	1
» » and magnamycin	14			1	3	5	5				
» » and erythromycin	14			1	3	1	1	4	1	2	1
» » and neomycin	14	1		1	4	8					
» » and tebezon	14		2		2	8	2				

Column A shows the number of eggs that died.
Column B shows the number of eggs that hatched.
Column C shows the number of eggs in which the chickens had picked only small holes in the shells.

TABLE 2

TOXICITY OF THE DRUGS

	No. of Eggs	Days after Inoculation									
		A								B	C
		2	4	5	6	7	8	9	10		
Buffer (pH 7.4)	11									10	1
» and sulfa	5									5	
» and magnamycin	9		1							7	
» and erythromycin ..	9			1				1	1	5	1
» and neomycin	9				1			1		6	1
» and tebezon	8		2							7	

Column A shows the number of eggs that died.
Column B shows the number of eggs that hatched.
Colum C shows the number of eggs in which the chickens had picked only small holes in the shells.

Discussion. — It is seen from Table 2 that erythromycin is somewhat toxic and from Table 1 that it has a slight effect on toxoplasmosis. Magnamycin, Neomycin and Tebezon are without effect on toxoplasmosis. Parts of the brains and livers of the chickens that hatched were inoculated intraperitoneally into mice, and Toxoplasma could be isolated from every one of them.

SUMMARY

Erythromycin has a weak effect on experimental toxoplasmosis in embryonated hen's eggs. The effect is not as good as that of sulfa. The chickens, however, remain Toxoplasma carriers. Magnamycin, Neomycin and Tebezon are without effect on toxoplasmosis.

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FROM THE DEPARTMENT OF ANATOMY, UNIVERSITY OF HELSINKI, AND FROM THE WIHURI RESEARCH INSTITUTE, HELSINKI

INACTIVATION OF THE THYROID IN EXPERIMENTAL OBSTRUCTION JAUNDICE

by

A. N. KUUSISTO and ANTTI TELKKÄ[1]

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Recently we reported (7) that the experimental obstruction jaundice obtained by the ligature of the common bile duct seems to have a depressing effect on the thyroid of rat. This effect is attributable to the fact that during an early stage of bile stasis there is, in blood, a great concentration of bile acids. Bile acids are cholesterol derivatives, many of which have been observed to possess a thyroid-inactivating effect. On the other hand, our experimental animals showed changes in other endocrines similar to those of Selye's general adaptation syndrome, suggesting that experimental obstruction jaundice can also be considered as a stress and the inactivation of the thyroid as the response to this stress.

In order to investigate this question, we performed a series of experiments using intact and adrenalectomized rats as test animals. Adrenalectomized animals can be regarded at least partially unable to respond to stress (5, 6). By this way it was thought to be possible, to a certain extent, to eliminate one of these two potential inactivating factors. Another way to elucidate this question, the administration of bile acid solutions to intact rats was in our experience impossible because of the great local toxicity of these substances.

[1] Aided by a grant from the Niilo Helander Foundation.

THE PRESENT INVESTIGATION

Forty male albino rats of the Wistar strain weighing 185—215 g were divided into four groups of ten animals. The first group served as controls, the rats of the second group were adrenalectomized bilaterally under evipan-sodium anaesthesia (Evipan-Natrium »Bayer»). Bilateral adrenalectomy was also performed to the rats of the third group; two weeks after this operation ligature of the common bile duct was made with double silk suture and the common bile duct was cut beneath the suture. To the rats of the fourth group the ligature of the common bile duct was performed in the same manner. The rats of the three first groups were killed by decapitation and bled out three weeks after the beginning of the experiment and those of the fourth group one week after the ligation. The adrenalectomized animals received 15 mg of crystalline desoxycorticosterone acetate (Percorten Kristall-Ampulle »Ciba») during the operation. The thyroid, pituitary, adrenal and thymus glands and the testes were removed and weighed. The thyroids were fixed in Bouin's fluid, embedded in paraffin and stained with Mallory's azan. The percental proportion of the thyroid epithelium, colloid and stroma was determined according to the method introduced by Uotila and Kannas (8).

RESULTS

A severe jaundice developed in all animals of the ligature groups within a few days. Maintenance of obstruction was determined at autopsy by the presence of a dilated, bile-filled duct proximal to the ligatures with no evidence of leakage. Several internal organs revealed a yellow pigmentation and the surface of the liver was very finely granular. The adrenalectomized rats were in a good condition except that a slight edema was noticeable in some of the cases.

The mean weights of the rats and their endocrine glands are shown in Table 1.

The differences in the weight of the thymus between the control group and the other groups were statistically significant. Also the differences of adrenal weight between the control and ligature group were statistically significant. The weight of the testes showed only possible differences between the different groups.

TABLE 1

	I Controls	II Adrenal-ectomized	III Adrenalec-tomized plus Ligature of the Com-mon Bile Duct	IV Ligature of the Common Bile Duct
Initial body weight (g)	201 ± 5	206 ± 2	205 ± 4	207 ± 7
Final body weight (g)	212 ± 5	210 ± 4	209 ± 5	180 ± 6
Weight of the thyroid (mg)....	20.8 ± 0.6	25.3 ± 1.9	24.3 ± 1.5	17.8 ± 1.1
Weight of the adrenals (mg)....	30.5 ± 1.1	—	—	51.0 ± 6.5
Weight of the pituitary (mg)....	6.8 ± 0.6	8.0 ± 0.2	7.5 ± 0.4	6.7 ± 0.3
Weight of the thymus (mg)....	190 ±14	355 ±13	358 ±18	104 ± 10
Weight of the testes (mg)....	2200 ±82	2348 ±77	2345 ±51	1968 ±157

In the animals of the ligature group histological examination of the adrenals revealed hypertrophy of the cortex; the thymus glands of this group showed an involution, and also in the testes a slight involution was noticeable. These changes of the thymus and the testes were not present in the adrenalectomized ligature group.

Histological examination of the thyroid of both the ligature groups showed an increased amount of the colloid and a low epithelium, i.e. a thyroid inactive as far as the histological picture is concerned (Figs. 1—3). The proportion of the epithelium, colloid and stroma as measured according to the method of Uotila and Kannas is given in Table 2. (The sum of the epithelium, colloid and stroma is denoted by 100).

The differences of the values of the epithelium and the colloid between the controls and the third and fourth group were statistic-

TABLE 2

	Epithe-lium	Colloid	Stroma
I Control animals	76.9 ± 1.8	16.1 ± 1.6	7.0 ± 0.7
II Adrenalectomized	76.3 ± 1.5	16.5 ± 1.5	7.2 ± 0.6
III Adrenalectomized plus ligature of the common bile duct	69.4 ± 1.3	22.0 ± 1.2	8.6 ± 0.9
IV Ligature of the common bile duct ..	64.9 ± 1.7	25.8 ± 1.9	9.3 ± 0.8

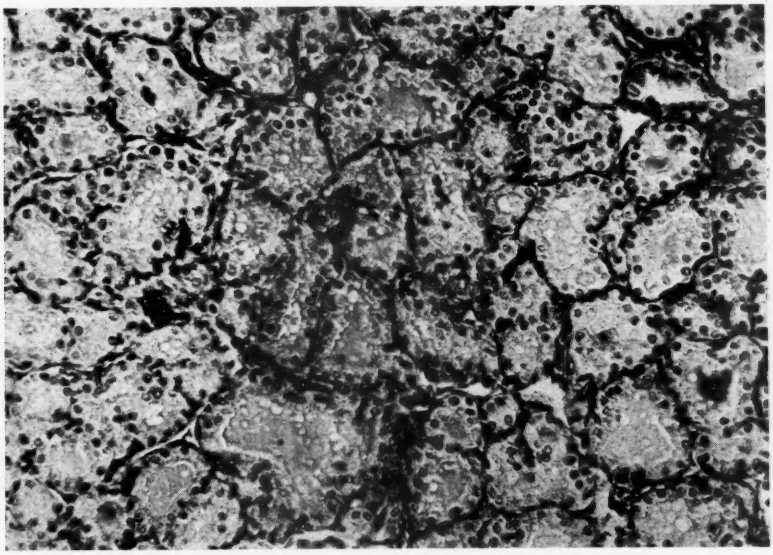

Fig. 1. — The thyroid of a control rat. 350 ×.

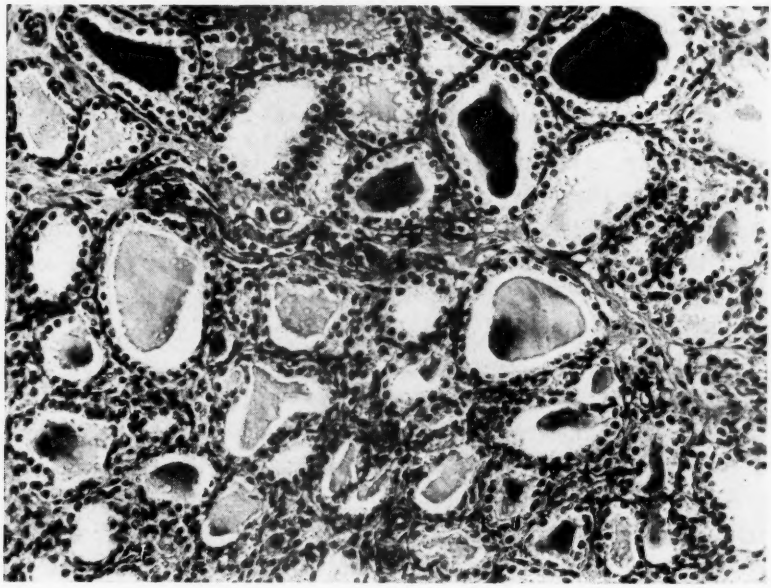

Fig. 2. — The thyroid of an adrenalectomized rat with the ligature of the common bile duct. 350×.

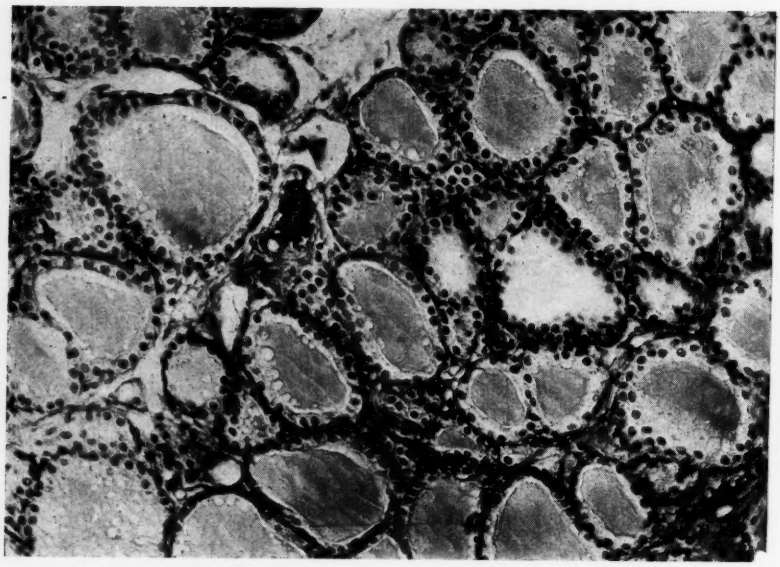

Fig. 3. — The thyroid of a rat with the ligature of the common bile duct. 350 ×.

ally significant, the differences of these values between the two last mentioned group were only possible. No difference was between the values of the controls and adrenalectomized animals.

DISCUSSION

According to our results, obstruction jaundice causes an inactivation of the thyroid of rat. This inactivation is present, if not so clearly as in intact animals, also after adrenalectomy. Therefore, it is to be assumed that obstruction jaundice itself has a thyroid-inactivating effect, which is probably due to the increased blood concentration of bile acids or to the recently demonstrated increased rate of synthesis of cholesterol in the liver following the ligation of the bile duct (1). Adrenalectomy seems to have a slight inhibiting effect on this inactivation. Thus, it is possible that the general adaptation syndrome plays a rôle in this inactivation and the decreased ability of adrenalectomized animals to react to stress also diminishes the inactivation of the thyroid. Our observations indicate that the principal depressing factor is the bile stasis and an additional factor may be the thyroid response to the stress due to this operation.

There are many discrepancies in the investigations concerning the response of the thyroid to stress, which is probably due to the fact that the thyroid response is usually bi-phasic and depends on conditioning factors (6). An inactivation or atrophy of the thyroid as a response to stress has been described by several authorities (e.g. 2—4). Our results seem to support this view.

Adrenalectomy alone in our experimental conditions did not affect the histological picture of the thyroid.

SUMMARY

The nature of the previously-observed thyroid-inactivating effect of the experimental obstruction jaundice was studied using intact and adrenalectomized rats as test animals. The inactivating effect of the experimental obstruction jaundice was present, if not so clearly as in intact animals, also after adrenalectomy. Therefore, it is to be assumed that the principal inactivating factor is obstruction jaundice with bile stasis and that the thyroid response to the stress due to this operation is an additional factor.

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FROM THE DEPARTMENT OF ANATOMY, UNIVERSITY OF HELSINKI, AND FROM THE WIHURI RESEARCH INSTITUTE, HELSINKI

EFFECT OF ALLOXAN ON THE THYROID GLAND OF INTACT AND ADRENALECTOMIZED RAT[1]

by

ANTTI TELKKÄ and A. N. KUUSISTO

(Received for publication July 27, 1954)

The appearance and the course of alloxan diabetes has been shown to be greatly modified by some endocrinological factors. Houssay, Martinez and their co-workers (9, 10, 15) demonstrated that thyroidectomized animals showed a greater resistance to the diabetogenic action of alloxan than the controls, whereas the treatment with powdered thyroid resulted in a resistance to alloxan below the normal after 20 days and above the normal after 60 days of daily treatment.

The adrenals also influence the alloxan diabetes. It has been shown that after adrenalectomy there is a marked reduction or a complete disappearance of diabetic symptoms in rats (12) which probably is in part associated with a lowered food intake of adrenalectomized animals (13).

The inverse relations, the influence of alloxan on the adrenals and on the thyroid, are relatively largely studied as regards the adrenals, but on the effect of alloxan on the thyroid there are only few investigations with somewhat diverging results. Earlier investigators often described the thyroid as normal in alloxan diabetes (2, 4). On the other hand, Bennett and Koneff (3) and Applegarth and Koneff (1) found an atrophy of the thyroid in alloxan diabetic rats.

[1] Aided by a grant from the Sigrid Jusélius Foundation.

The present study was undertaken in an attempt to find out whether the thyroid is influenced by alloxan, as measured by a histoquantitative method. Because the role of the adrenals in the appearance and course of alloxan diabetes, besides intact animals, also adrenalectomized rats were used. Observations were also made on some other endocrines.

MATERIAL AND METHOD

The initial series consisted of 50 male albino rats of the Sprague-Dawley strain weighing about 100 g. Some animals from the experimental groups died during the experimental period and the final number of the rats of each group is given in Table 1. The rats were subdivided into four groups, the first of them serving as controls. To the rats of the second group alloxan monohydrate (British Drug House, London) was given in a single subcutaneous injection of 150 mg/kg of body weight. The rats of the third group were adrenalectomized bilaterally under combined hexobarbitone-aether anaesthesia. Immediately after the operation they received alloxan monohydrate subcutaneously also in a dose of 150 mg/kg of body weight. The rats of the fourth group were adrenalectomized bilaterally in a similar manner as those of the third group. The rats which received alloxan were fasted 24 hours before injection. After the operation and administration of alloxan the rats were provided with a diet which consisted of: oat meal 24%, ground whole oat 24%, ground whole wheat 24%, rye meal 24%, brewer's yeast 1.3%, calcium lactate 1.3%, cod liver oil 1%, and sodium chloride 0.4%. The diet was restricted for the controls whereas the experimental animals were permitted to eat ad libitum.

After seven days the rats were killed by decapitation without anaesthesia and bled. They were fasted seven hours before the sacrifice for the blood sugar determination, which was performed using the method of Hagedorn and Jensen (7). Blood specimens were taken just prior to sacrifice. The thyroid, adrenal and thymus glands, and the testes were removed and weighed. They were fixed in Bouin's fluid, imbedded in paraffin and stained with Mallory's azan method. The percental proportion of the thyroid epithelium, colloid and stroma was determined from a projected picture of the specimen employing the histoquantitative method introduced by Uotila and Kannas (19). Pieces from the pancreas were stained with the method of Scott (16) for the beta-cells of pancreatic islets.

RESULTS

The mean initial and final body weights of the rats, the mean weights of their endocrines and the mean blood sugar values are tabulated in Table 1.

TABLE 1

	I Controls (n=10)	II Alloxan Injected (n=11)	III Adrenal-ectomized plus Alloxan Injected (n=11)	IV Adrenal-ectomized (n=12)
Initial body weight (gm)	100 ± 6	97 ± 7	106 ± 7	100 ± 6
Final body weight (gm)	112 ±10	95 ± 8	115 ±10	113 ± 8
Weight of the thyroid (mg)	13.6 ± 0.9	12.6 ± 1.1	15.5 ± 1.3	15.7 ± 1.1
Thyroid weight/final body weight (mg/g)	12.1 ± 4.6	13.3 ± 5.0	13.5 ± 5.2	13.8 ± 4.4
Weight of the adrenals (mg)	25.0 ± 1.1	24.0 ± 0.9	—	—
Adrenal weight/final body weight (mg/g)	22.3 ± 5.1	26.3 ± 4.2	—	—
Weight of the thymus (mg)	267 ±11	183 ±14	406 ±16	478 ±14
Weight of the testes (g)	1.71± 0.1	1.54± 0.1	1.79± 0.1	1.76± 0.1
Blood sugar (mg%)	96.5 ± 5	396 ±22	111 ± 8	110 ± 8

From the organ weights presented only the differences between the thymus weights of all four groups were statistically significant. The mean relative weights of the thyroids and the mean relative weight of the adrenals showed no statistically significant differences between the different groups.

The mean blood sugar value of the alloxan injected group differred significantly from those of the three other groups. In the alloxan injected group the lowest blood sugar value was 150 mg per 100 ml and the highest 645 mg per 100 ml. According to Houssay et al. (11) the rat can be considered to be diabetic if the blood sugar is above 150 mg per 100 ml after seven hour fast. The highest blood sugar value in the other groups was that of 135 mg per 100 ml in a rat of the adrenalectomized group.

The percental proportion of the thyroid epithelium, colloid and stroma as determined according to the method of Uotila and Kannas was as follows:

	Epithelium	Colloid	Stroma
Control rats	81.9 ± 1.6	10.9 ± 1.1	7.2 ± 0.4
Alloxan injected rats	71.8 ± 1.4	19.8 ± 1.0	8.4 ± 0.6
Adrenalectomized plus alloxan injected rats	77.7 ± 1.9	15.7 ± 1.2	6.6 ± 0.4
Adrenalectomized rats	81.7 ± 1.5	12.3 ± 1.9	6.0 ± 0.5

The differences between the values of both the epithelium and the colloid were statistically significant between the controls and the alloxan injected group. Also the values of the adrenalectomized group differred significantly from those of the alloxan injected group. The severity of the diabetes and the inactivation of the thyroid seemed to be closely correlated, the most flattened thyroid epithelium and the highest blood sugar values were noticeable in the same animals.

Beta-cells of the pancreatic islets in the alloxan injected rats were nearly completely disappeared, whereas they were substantially unaltered in the other groups.

DISCUSSION

According to our results, alloxan diabetes resulted in an inactivation of the thyroid of rats, which was demonstrable by the histoquantitative method used. This inactivation seemed to be directly correlated to the severity of the diabetes. This result is in agreement with that presented by Bennett and Koneff (3) and Applegarth and Koneff (1), although we were unable to found any statistically significant changes in the weights of the thyroids. Whether this inactivation is a direct result of the action of alloxan on the thyroid or whether it is secondary to the deranged metabolism of the diabetic animal, must be discussed. Bennett and Koneff believed that the changes in the thyroid were secondary to the resulting diabetes because they found that insulin largely prevented the thyroid changes and, in addition, in the thyroids of alloxan diabetic animals acute necrotic lesions of the type that alloxan produces elsewhere were not found. Our experiments with the adrenalectomized plus alloxan injected rats support their view. The administration of alloxan did not affect the thyroid without concomitant outbreak of the diabetes. Furthermore, the severity of the diabetes and the inactivation of the thyroid seemed to be directly correlated in our experiments. The inactivation of the thyroid has also been observed in experimental diabetes obtained by subtotal pancreatectomy (5). Adrenalectomy, as we previously could establish, did not modify the activity of the thyroid (14).

In our experimental conditions, adrenalectomy seemed to prevent the outbreak of the diabetes. This is in agreement with the

observations of Janes and Friedgood (12). These results confirm that the adrenals play a considerable role in the mechanism of alloxan diabetes. According to Goldner and Gomori (6) adrenalectomy or demedullation of the adrenals prevented also the initial hyperglycemic phase due to alloxan, but a severe fatal initial hypoglycemia developed, probably the lack of adrenal counter-regulation enhanced the fall of the blood sugar. We did not determine the blood sugar at the time of the initial hypoglycemia, but the mortality in the adrenalectomized plus alloxan injected group was the greatest and the animals died with convulsions about ten hours after the alloxan injection.

Since the changes especially in the adrenals in alloxan diabetes are similar to those of Selye's adaptation syndrome, he emphasized (17, 18) that alloxan — like so many other drugs — can be considered to be a stressor agent. In our series we could not found any changes in the adrenals characteristic to this syndrome, but the mean weight of thymus glands was significantly below that of controls, another sign which is characteristic to the alarm reaction.

The mean weights of the thymus glands in both the adrenalectomized groups were above to those of controls, a finding which appears in general in adrenalectomized animals.

SUMMARY

The influence of alloxan on the thyroids of intact and adrenalectomized rats is studied. In the thyroids of intact rats alloxan resulted in an inactivation of the thyroid. Adrenalectomy prevented the outbreak of diabetes and the thyroids remained unchanged as compared with those of the controls. Thus, our results indicate that the administration of alloxan did not directly affect the thyroid and that the inactivation of the thyroid is probably secondary to the deranged metabolism of the diabetic animal.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

THE MNS BLOOD GROUPS IN FINLAND

by

O. MÄKELÄ

(Received for publication April 5, 1954)

The MN blood group system was found by Landsteiner and Levine (2, 3, 4, 5) in 1927. The system appeared to be very solid and clear. The occurrence of the two antigens M and N depended on two mutually allelomorphic genes, each of which manifested itself even in single dose. Thus the genotype MN could be clearly demonstrated. This state of affairs continued until 1947, when Walsh and Montgomery (12) discovered an unknown antibody in human serum. Sanger and Race (9) proved that the antigen (which they chose to call S) corresponding to the new agglutinin, and the MN antigens were interdependent. Following quite extensive statistical and genetic researches two alternatives remained: either four genes M, N, MS, and NS instead of two were contained at the MN locus or the MNS system depended upon two pairs of very closely coupled genes. In the latter case the existence both of the fourth gene allelomorphic to S and of the corresponding antigen had to be assumed. This alternative was regarded as the more likely of the two. An intensive and, above all, purposeful study of the MNS blood groups that took four years was launched. The missing fourth antigen was named s. Human immunisations, for instance, were carried out on a rather large scale in order that the corresponding agglutinin might be found — all without avail. It was estimated that 89 per cent of the blood specimens of Englishmen would be agglutinated by it provided it could ever be found. It seemed as if, in such a case, the MNS system would prove the most useful in the study of twins and in the exclusion of paternity. In April, 1951, anti-s was found at last in the blood of a mother who had given birth to a hemolytic child (6).

The serum came up to expectations in every way. It agglutinated 89.09 per cent of the blood samples of Englishmen and detected s even in single dose just as anti-M, anti-N and anti-S did. Thereafter the MNSs system was quite as solid and clear as twenty years before, only twice as large.

The close linking of MN and Ss genes makes them eminently suitable, theoretically at least, for anthropological studies. In addition to MN and Ss gene frequencies it is possible to make use of MS, Ms, NS, and Ns chromosome frequencies, since, because of the linkage, e.g. the proportion MS:Ms remains constant in a given population and usually differs from S:s.

Dr. Mourant kindly supplied the Department of Serology and Bacteriology, University of Helsinki, with a small quantity of the anti-S serum, by means of which I have been able to check 217 random samples of blood from Finnish persons with regard to the MNSs groups.

METHODS

The MN characteristics were each determined on a slide by means of two sera; the results were uniform. In order to spare the serum, the S characteristics were determined in small test tubes. The reaction was somewhat vague in two cases only. Repeated tests proved these samples to be positive, too.

The gene and chromosome frequencies were calculated by means of the maximum likelihood method.

TABLE 1

RESULTS

THE MNS BLOOD GROUPS IN FINLAND

Phenotype	Number Observed	Frequency Observed	Chromosome	Frequencies	Number Expected
MS	60	.27649	MS	.24882	59.673
Ms	42	.19355	Ms	.42819	39.786
MNS	51	.23502	NS	.08953	51.515
MNs	39	.17972	Ns	.23347	43.387
NS	11	.05069	Total	1.00001	10.811
Ns	14	.06452			11.828
Total	217	.99999			217.000

$\chi^2 = 0.976$,
two degrees of freedom

DISCUSSION

The MN gene frequencies of our series (M = .67701, N = .32300) seem to coincide, on the whole, with those frequencies for Finns that can be calculated on the basis of the large combined material of Mustakallio (7) and Streng (11) (M = .643, N = .357).

Since anthropological studies employing the MNSs groups are of a recent origin and the material is still limited, there seems to be no reason for a general discussion. In Table 2, only two series of

TABLE 2

SOME GENE AND CHROMOSOME FREQUENCIES

Population	Number Tested	Gene Frequencies				Chromosome Frequencies			
		M	N	S	s	MS	Ms	NS	Ns
Englishmen	1419	.53030	.46970	.32738	.67262	.24717	.28313	.08021	.38949
Norwegian Lapps	183	.5164	.4837	.3685	.6316	.1881	.3283	.1804	.3033
Finns	217	.67701	.32300	.33835	.66165	.24882	.42819	.08953	.23347

frequencies are included; the hitherto largest material from Englishmen (8) and the hitherto only Scandinavian material from the Norwegian Lapps (1). It is interesting to compare the frequencies of English persons on the one hand and of Finnish persons on the other. Ss frequencies are very much alike, whereas MN frequencies differ. Moreover, with Englishmen S is very markedly accumulated by the M gene, s by the N gene, while with Finns this tendency is much less pronounced. Thus, with English persons the proportion MS:Ms is equal to .87 and with Finnish persons .58; the respective proportions NS:Ns are .21 and .38.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

AN ERYTHROCYTE SEDIMENTATION METHOD FOR DETECTING INCOMPLETE ANTIBODIES *

by

O. MÄKELÄ and PIRJO MÄKELÄ

(Received for publication July 21, 1954)

The detection of incomplete antibodies is no very simple task. Even the albumin agglutination method regarded as the easiest and most practical presupposes, in addition to albumin, a thermostat and a microscope, a person with some experience in reading albumin agglutination. Several other methods are either far more complicated or less reliable. Thus, the detection of immune antibodies has been taken up by some few central laboratories, to which the samples may have to be sent across quite great distances.

Of the colloids used as adjuvants in the determination of incomplete antibodies, those have proved the most effective, which as such bring about a pronounced rouleaux formation and consequently increase the sedimentation rate (3). Thus dextran has been regarded as very practical (2). According to our experience, however, agglutinations obtained in dextran milieu are difficult to read because of the strong rouleaux formation.

The incomplete antibodies coupled to cells were demonstrated by Blessing and Heni in 1953 with the aid of the SR technique (1). They noticed that if the red cells of a patient were directly agglutinated by anti-human globulin serum, a very rapid sedimentation took place in a medium containig dextran. The number of such patients was 3, that of totally negative controls 100, among whom

* Presented at the meeting of the Finnish Society of Pathologists, Turku, May 8, 1954.

there were normal persons and patients with various maladies whose ordinary SR was high.

These scientists came to the conclusion that when cells were saturated with incomplete antibodies, the agglomeration was pronounced.

It occurred to us that incomplete blood group antibodies in serum could be detected with a somewhat similar technique instead of estimating the intensity of the agglomeration on a slide.

MATERIAL AND METHODS

We examined some forty sera, half of which were known to contain incomplete Rh antibodies. All positive sera were titrated with the SR technique as well as the albumin, Coombs and papain methods. In all experiments the same O R_1R_2 cells were used.

Of the test cells, which were washed two or three times, 0.5 cc was added into 2 cc of the serum to be examined. After half an hour's incubation at 37°C the cells were washed twice. The packed cells were mixed with 1 cc of 6% dextran and the mixture was sucked into Westergren pipettes, in which the SR was read after one hour.

The readings were of three main types (Fig. 1): 1.) The upper end of the cell column was diffuse; the sedimentation rate, ascertainable with some difficulty, was 35 to 50 mm. This was a typical negative reading. 2.) The upper end of the cell column was diffuse, but a distinct step was seen, and the SR derived from it was 100 to 120 mm. 3.) The upper end of the cell column was abrupt and the agglomerates were clearly discernible to the naked eye. Types 2. and 3. occurred when appropriate antibodies were present in the serum examined. The resulting agglomeration could be so pronounced as to impede sedimentation; pellucid spaces could appear even within the cell column, also when normal SR pipettes with a diameter of 2 mm were used.

It was found, when experimenting with the technique, that by using 0.1 cc of cells instead of 0.5 cc, the method became more sensitive. Reading, however, became slightly more difficult, for in a more dilute cell suspension the sedimentation is quicker; and even the negative controls show a high SR. Thus, when reading the results, attention must be paid to the upper end of the cell column only. In negative cases it is diffuse, in positive ones with this modification always abrupt.

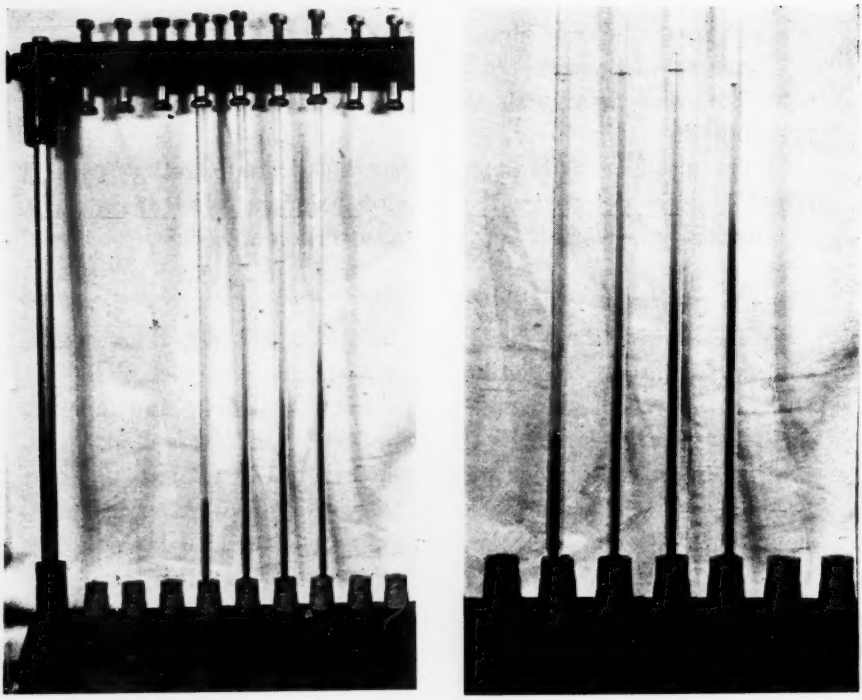

Fig. 1. — Sedimentation reactions of various kinds. Pipettes from the right hand on: negative reaction, intermediate type, weak and strong positive reactions.

The medium used was, besides mere 6% dextran, dextran + 0.9% NaCl as used by Blessing and Heni. Our method was found to be more sensitive and equally reliable. Very stable readings were obtained by using dextran + human AB serum as a medium but an addition of serum, too, results in a decrease of sensitivity.

The incubation temperature of 37° C was adopted from the Coombs technique. Five sera, however, were titrated with the SR method and with indirect anti-human globulin method at 18° C, as well as at 37° C. In both techniques the results obtained at room temperature were similar to those obtained at 37° C.

When varying the duration of the incubation period we noticed that the coupling of antibodies with receptors was fairly complete at least in ten minutes. Lewi has come to the same conclusion examining isoagglutinins (4.)

The incubated cells were washed three times lest the general characteristics of the serum should influence the results. This necessitated many centrifugations and made the technique unduly laborious. Therefore, we have made parallel determinations with cells washed not at all to 3 times using different sera, among them some with a high sedimentation rate. The results have been very nearly the same. Thus, only one centrifugation should be needed in order to remove the serum examined.

RESULTS

Of the 40 sera examined, 20 were such as could not be shown to contain immune antibodies with usual methods. The result obtained with the aid of the SR technique was negative, too.

The remaining 20 were such as with some usual method could be shown to contain Rh antibodies and were Rh negative. In all

TABLE 1

TITRES OF 20 RH IMMUNE SERA WITH VARIOUS TECHNIQUES

Serum	Titre as 1/n			
	SR	Albumin	Coombs	Papain
1.	—	1	—	—
2.	—	2	4	4
3.	—	4	—	64
4.	8	16	16	4
5.	16	16	32	256
6.	64	64	16	128
7.	64	64	64	256
8.	3200	3200	800	25600
9.	4	1	—	—
10.	4	2	64	32
11.	8	2	1	1
12.	8	4	1	8
13.	16	1	1	32
14.	32	1	16	128
15.	64	4	32	128
16.	128	16	4	64
17.	400	200	800	1600
18.	800	100	100	16
19.	800	100	100	3200
20.	3200	1600	3200	25600

these cases there were parturitions or blood transfusions in the anamnesis. The use of Rh negative cells as control cells did not yield positive results in a single case. Titration results are in Table 1.

DISCUSSION

When considering the usefulness of the SR technique on the basis of the above results, one must say at once that it seems to be specific. The sensitivity can be judged only after large numbers of samples have been examined and after there have been possibilities to find antibodies by means of it in sera giving negative results with other methods. Actually, the sensitivity of the SR technique must be inferred from a comparison of the titres. In three sera the presence of antibodies could not be demonstrated with the SR method. All of these were weak with other techniques. There was one serum, the titre of which lagged behind the albumin titre, four sera where the titres were equal, and twelve with which the SR method seemed to be more sensitive. No sera were positive with the SR technique and negative in other tests, owing to the fact that merely albumin positive sera were examined. Anti-human globulin and papain titres went farther, as a rule.

A technical drawback of the SR method is its laboriousness, an advantage is its easy interpretability e.g. by nurses. It involves the need of much serum and thus cannot be used to determine Rh groups. It might prove useful to obstetrical practice in out-of-the-way regions as the only method of determining immunisation. No examination of the patients' cells would then be undertaken.

SUMMARY

A method is described of how incomplete antibodies can be demonstrated by utilising the sedimentation in 6% dextran of test cells saturated with antibodies.

It is observed that the method is specific and approximately equal to the albumin agglutination method in sensitivity. It is rather laborious, while being extremely easy to read even by an unaccustomed technician.

The quite prompt fixation of incomplete antibodies to receptors is noted as well as the relative independence of coupling on temperature.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI, AND FROM THE FIRST SURGICAL UNIVERSITY CLINIC, HELSINKI

l-USNIC ACID, DIHYDROSTREPTOMYCIN, AND THEIR COMBINATION

THERAPEUTIC EFFECT IN GUINEA-PIG TUBERCULOSIS AS REVEALED BY THE OMENTAL INDEX

by

JORMA PÄTIÄLÄ and ELJAS BRANDER

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Eung Soo Han and others (1) presented a quick method of studying the effect of different drugs on experimental guinea pig tuberculosis. Inoculation with tuberculosis was performed intraperitoneally, antituberculosis treatment being started at the same time. At the end of two weeks the greatly swollen omentum was removed and weighed. Care was taken to exclude the pancreas, which, in the guinea pig, fans out in the omentum from the region of the splenic hilum. The ratio of the weight of the omentum in grams to the weight of the guinea pig in kilograms has been called the omental index. By means of this index it was possible to evaluate the effect of the drug employed. If an effective antimicrobial is used, the omentum increases in weight very little and the omental index remains near the normal level.

The above-mentioned index has been used in the present work to study the antituberculosis effect of l-usnic acid and the combined treatment with l-usnic acid and streptomycin.

MATERIAL AND METHODS

Twenty guinea pigs, weighing approximately 500 g, were intraperitoneally inoculated with 0.2 mg of virulent tubercle bacilli

($H_{37}Rv$) suspended in 0.1 ml of Dubos fluid medium. The weight of the bacilli was determined by means of Hopkins vaccine tube.

For the purpose of treatment with drugs, the animals were divided in five groups of five. Group One or the control guinea-pigs received no treatment. Group Two was given 20 mg of l-usnic acid per os daily (product of Lääketehdas Orion). Group Three was administered 3 mg of dihydrostreptomycin a day subcutaneously like Group Four, but the latter was also given 20 mg l-usnic acid through the mouth in the same way as Group Two. Group Five received 6 mg of isonicotinic acid hydrazide (Rimifon »La Roche») subcutaneuously once a day.

Treatment with drugs was started in groups Two to Five on the day of the inoculation with tuberculosis, and continued over two weeks. After that the guinea pigs were put to death and dissected, the omenta were cautiously removed, the pancreas being separated from them, after which the omenta were weighed and the omental index determined.

RESULTS

The results are to be seen in Fig. 1 and Table 1.

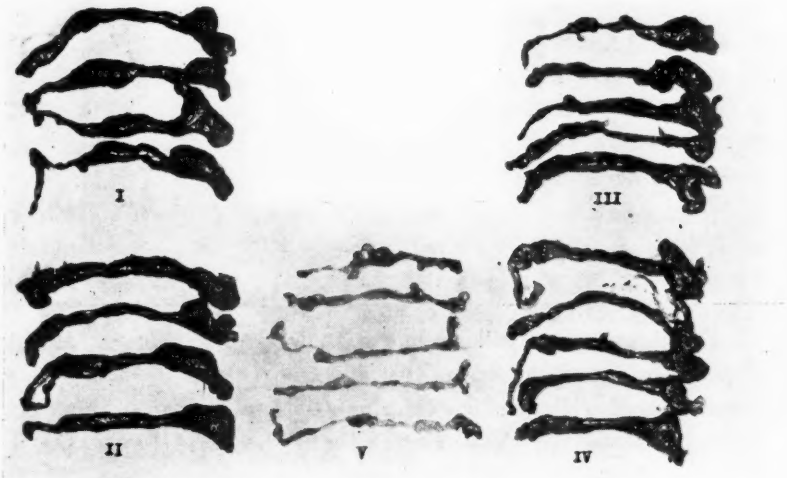

Fig. 1. — (I) Infected controls. — (II) l-Usnic acid, 20 mg orally. — (III) Dihydrostreptomycin, 3 mg subcutaneously. — (IV) Dihydrostreptomycin, 3 mg subcutaneously, and l-usnic acid, 20 mg orally. — (V) Isonicotinic acid hydrazide, 6 mg subcutaneously.

TABLE 1

THE THERAPEUTIC EFFECT OF L-USNIC ACID, DIHYDROSTREPTOMYCIN AND OF ISONICOTINIC ACID HYDRA IDE AND OF THEIR COMBINATIONS AS REVEALED BY THE OMENTAL INDEX

Group	Animal Number	Therapy	Weight of Pig	Weight of Oomentum	Omental Index	
					Individual	Average
I	1	Infected control	0.660	4.60	7.0	
	2	—»—	0.500	2.90	6.0	
	3	—»—	0.540	5.30	9.8	
	4	—»—	0.520	5.00	9.6	8.1
	5	—»—	failure			
II	6	l-Usnic acid 20 mg	0.450	4.82	10.7	
	7	—»—	0.460	4.27	9.2	
	8	—»—	0.560	5.65	10.0	
	9	—»—	0.480	4.18	8.7	
	10	—»—	0.440	4.58	10.4	9.8
III	11	Dihydrostreptomycin 3 mg	0.510	2.17	4.2	
	12	—»—	0.630	2.25	3.5	
	13	—»—	0.630	3.65	5.8	
	14	—»—	0.520	2.20	4.2	
	15	—»—	0.480	3.80	8.0	5.1
IV	16	Dihydrostreptomycin 3 mg+l-Usnic acid 20 mg	0.540	2.51	4.6	
	17	—»—	0.530	2.03	3.8	
	18	—»—	0.560	4.10	7.3	
	19	—»—	0.470	2.60	5.5	
	20	—»—	0.520	4.01	7.7	5.7
V	21	Isonicotinic acid hydrazide 6 mg	0.540	0.49	0.9	
	22	—»—	0.510	0.71	1.4	
	23	—»—	0.580	0.50	0.8	
	24	—»—	0.490	0.47	0.9	
	25	—»—	0.510	0.75	1.4	1.0

The figure alone will suffice to show that the largest omenta were to be found in the control animals (Group I) as well as in those treated with l-usnic acid alone (Group II). According to the table the omental indexes were 8.1 and 9.8 respectively. On the other hand, the omenta of the animals that had been given dihydrostreptomycin alone (Group III) were clearly less swollen than in the first two groups, the omental index also being lower, viz. 5.1. The animals that had received l-usnic acid along with dihydro-

streptomycin (Group IV) had omenta approximately of the same size as those treated only with dihydrostreptomycin, the index too being the same. — The smallest omenta and the lowest omental index occur in Group V, which received isonicotinic acid hydrazide. The daily dose of isonicotinic acid hydrazide of 6 mg was made that big in order to secure another point of comparison as a contrast to the control guinea pigs for observing an intensified chemotherapeutic effect. Karlson and Feldman (3) have namely found out that as small a dose of isonicotinic acid hydrazide as 1 mg was enough almost completely to prevent the development of tuberculosis in guinea pigs. It is true, the technique used by them was somewhat different.

DISCUSSION

The above series of experiments shows that in spite of the positive results earlier achieved with l-usnic acid in the treatment of guinea pig tuberculosis (2, 4, 6, 7), a daily dose of 20 mg per os during two weeks could not prevent tuberculosis from developing judged by omental index. It is obvious that the properties of this agent whose antituberculosis effect is comparatively weak, cannot be demonstrated by means of the technique used in the present experiment, in which the therapeutic exposition period is short and the inoculum of bacilli comparatively high. But in the animals that were given 3 mg dihydrostreptomycin the omental tuberculosis was considerably less severe than in the control animals and those treated with usnic acid, though it is not comparable with the isnonicotinic acid hydrazide group, where the antituberculotic effect was very strong. When l-usnic acid (20 mg) and dihydrostreptomycin (3 mg) were given simultaneously, the result was the same as with dihydrostreptomycin alone. This finding differs considerably from the observations by Marshak and Kuschner (5), according to whom a comparable, marked retardation in the development of the disease was obtained with high doses of streptomycin (6 mg a day), and with usnic acid (20 mg) combined with low doses of streptomycin (2 mg a day), these low doses of streptomycin alone effecting no retardation of the disease. However, the experiments performed by Marshak and Kuschner are so different from ours as regards the methods that they cannot be looked upon as fully comparable.

SUMMARY

The writers have studied the effect of l-usnic acid on the development of guinea pig tuberculosis both separately and together with dihydrostreptomycin. An intraperitoneal technique of inoculation was employed, and the so-called omental index was used in order to evaluate the effect of the drugs after two weeks' treatment with the result that the tuberculostatic effect of l-usnic acid could not be brought out.

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FROM THE DEPARTMENT OF FORENSIC MEDICINE, UNIVERSITY OF HELSINKI, AND FROM THE UNIVERSITY DEPARTMENT OF THE HELSINKI TUBERCULOSIS HOSPITAL

REACTION OF THE THYROID TO EXPERIMENTAL TUBERCULOSIS IN GUINEA PIGS

by

JORMA PÄTIÄLÄ and ANTTI ISOTALO

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For more than a hundred years it has been considered probable, on the basis of clinical observations, that tuberculosis has a certain relationship with thyroid function. The most important conclusion made by investigators in this connection is the frequent presence, in the early stages of tuberculosis, of symptoms pointing to increased thyroid activity, whereas in the more chronic forms of tuberculosis there frequently is atrophy and hypofunction of the thyroid gland (2, 4, 6, 7). Experimental studies have pointed to the same conclusion (12, 14).

The study of the relationship between tuberculosis and the thyroid gland has gained new significance in the light of Selye's adaptation theory, and in recent years the part played by the thyroid in the general adaptation syndrome has received increased attention (1, 8, 11, 13).

It was the object of this investigation to study the occurrence of the stress syndrome in experimental guinea pig tuberculosis by determining the effect of tuberculosis infection on the histoquantitative picture of the thyroid gland and on the weight of the thyroid, liver, spleen, kidneys, adrenals and testes. Comparison may be made between these results and the behaviour of the thyroid gland in other conditions of stress studied by the histoquantitative

method (5, 9). To our knowledge this method has not been used earlier in the study of the stress syndrome in tuberculosis.

MATERIAL AND METHODS

Two groups of adult male guinea pigs, each consisting of 10 animals, were used in this experiment. The first group was subcutaneously inoculated in the inguinal region with 0.1 mg of virulent tubercle bacilli, strain H_{37} Rv. The second group served as controls. Eight weeks later the animals were killed by decapitation, and the thyroid gland was dissected as quickly as possible, weighed and fixed in Bouin's solution. The liver, spleen, kidneys, adrenals and testes were also weighed. Histological preparations were made of the thyroid gland by staining with Koneff's modification of Mallory's azan method, and quantitative measurement of the histological components of the gland was carried out by the linear measurement method of Uotila and Kannas (1952). The percentages of epithelium and colloid in the gland were calculated on the basis of these measurements and the average values for the two groups were compared by statistical methods.

RESULTS

Both experimental groups of animals were in a relatively good condition 8 weeks after inoculation, although tuberculosis had developed in the animals in the first group. Macroscopically and microscopically there were tuberculous foci in the spleen and the

TABLE 1

ABSOLUTE BODY AND ORGAN WEIGHTS IN TUBERCULOSIS AND CONTROL GROUPS (GROUP AVERAGES)

	Body weight (gm)	Thyroid (mg)	Liver (mg)	Spleen (mg)	Kidneys (mg)	Adrenals (mg)	Testes (mg)
Controls	613	89	20348	684	3930	292	4010
	±28.2	±5.9	±815	±31.2	±159	±42.1	±169
Tuberculosis group	686	88	32465	1896	4755	474	3427
	±37.0	±5.1	±1374	±260	±254	±59.0	±307
Difference	+73	—1	+11117	+1212	+825	+182	—583
Confidence level	no	no	99.9%	99.9%	no	98%	no

lungs and in one case also in the liver. Table 1 shows the weights of the dissected organs at autopsy. Since the body weights of the animals were greatly variable, the weight of each organ in relation to body weight was calculated in mg per 100 gm. These relative weights are listed in table 2.

TABLE 2

RELATIVE WEIGHTS OF ORGANS IN TUBERCULOSIS AND CONTROL GROUPS (GROUP AVERAGES IN MG PER 100 GM BODY WEIGHT)

	Thyroid	Liver	Spleen	Kidneys	Adrenals	Testes
Controls	14.5	3357	113	660	46.9	643
	±0.69	±142	± 6.4	±11.8	±4.56	±27.2
Tuberculosis group	13.1	4816	285	696	68.4	500
	±1.01	±2.29	±42.5	±19.7	±6.60	±38.1
Difference	—1.4	+1459	+172	+36	+21.5	—143
Confidence level ..	no	99.9%	99.9%	no	99.1%	99.2%

It will be observed that the average weights of the thyroid gland in the tuberculosis and control groups showed no significant difference. The liver, spleen and adrenals, on the other hand, were definitely increased in weight in the tuberculosis group, whereas the testes had decreased in weight. No difference was evident in the weight of the kidneys.

Table 3 shows the results of the histoquantitative measurement of the thyroid gland, expressed as average group percentages of epithelium, colloid and stroma in the gland. The standard errors, the differences between the two groups, and the confidence limits are also shown.

TABLE 3

HISTOQUANTITATIVE MEASUREMENTS OF THE THYROID GLANDS IN TUBERCULOSIS AND CONTROL GROUPS (AVERAGE GROUP PERCENTAGES OF EPITHELIUM, COLLOID AND STROMA)

	E%	C%	S%
Controls	64.3±1.53	31.7±1.54	4.0±2.6
Tuberculosis group	58.2+1.43	39.2+1.39	2.6+0.32
Difference	—6.1	+7.5	—1.4
Confidence level	99.1%	99.9%	96%

A definite difference will be observed between the tuberculosis and control groups in the proportion of all components of the thyroid gland (d E% —6.7, d C% +7.5, and d S% —1.4). Histological examination thus reveals inactivation of the thyroid gland in the group of guinea pigs inoculated with tuberculosis.

DISCUSSION

Eight weeks after inoculation with tuberculosis bacilli all the infected animals showed tuberculous changes in the spleen and lungs, and one animals in the liver also. The animals were nevertheless in a fairly good condition. The typical stress syndrome was evident in all the infected animals as an increase in the absolute and relative weights of the adrenals and the liver as well as signs of inactivation in the histoquantitative picture of the thyroid gland. As was seen in table 1—3, the group averages for adrenal and liver weights and for the histoquantitative measurements of the thyroid show a statistically significant difference between the control group and the animals inoculated with tuberculosis. The spleen was also significantly increased in the latter both absolutely and relatively. This is ascribable chiefly to the specific effect on the spleen of tuberculosis infection, which, as is well known, may cause a very marked increase in spleen weight. Similarly, the decrease in the relative weight of the testes of the infected guinea pigs in this study may be the result of the specific effect of tuberculosis infection. Elo (1945), among others, has observed that the ovaries of women who have died of pulmonary tuberculosis frequently show atrophic and degenerative changes. As to the effect of tuberculosis infection on the thyroid gland, the results obtained in our test series point chiefly to a non-specific effect, caused mainly by a general reaction of the organism against infection. As Botkin and Jensen have also stated in connection with one of their studies, it is difficult to demonstrate whether the histological inactivation of the thyroid such as was seen in this examination indicates also a functional inactivation of the gland. This study, furthermore, does not indicate whether histological activation of the thyroid occurs in the early stage of tuberculosis infection, as is usual in the beginning of the stress syndrome (11).

SUMMARY

Experiments on the reaction of the thyroid gland to tuberculosis were carried out with 20 guinea pigs, 10 of which were infected with 0.1 mg of virulent tubercle bacilli of strain H_{37} Rv. Eight weeks after inoculation the quantitative changes in the histological picture of the thyroid gland and alterations in the weights of the thyroid, liver, spleen, kidneys, adrenals and testes were determined. The histoquantitative examination showed a decrease in the percentage of epithelium in the thyroid (d E%—6.7) as a sign of inactivation of the gland as compared with the non-infected control animals. The weights of the liver, spleen and adrenals were significantly increased over the control group weights, and the testes showed a reduction in the relative weight. On the other hand, no difference was seen in the absolute or relative weights of the thyroid gland and the adrenals and in the total body weights of the animals in the infected and control groups. On the basis of these results and those of other workers in other conditions of stress, the writers conclude that the effect of tuberculosis infection on the inoculated animals was non-specific, being chiefly a result of the general reaction of the organism against infection.

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FROM THE DEPARTMENT OF ANATOMY AND THE WOMEN'S CLINIC, UNIVERSITY OF HELSINKI

HISTOCHEMICALLY DEMONSTRABLE SUCCINIC DEHYDROGENASE ACTIVITY IN PLACENTAL TISSUES

by

ANTTI TELKKÄ and LAURI LEHTO

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In their extensive series of studies Wislocki, Dempsey and their associates have largely investigated the histology and histochemistry of mammalian placenta (7—11). By histochemical means, it seems possible to throw some additional light on the function and physiology of the placenta. The aim of the present study is to investigate the histochemically demonstrable succinic dehydrogenase activity of the placental tissues.

Succinic dehydrogenase is an important link in the chain of enzymes involved in the citric acid cycle of Krebs which are responsible for biologic oxidation. Seligman and Rutenburg (6) have introduced a histochemical method for the demonstration of succinic dehydrogenase activity. In this reaction, tetrazolium salts serve, instead of the natural cytochromes, as hydrogen acceptors for succinic dehydrogenase. In the presence of succinate, the soluble tetrazolium salts are reduced to purple, water-insoluble formazan granules at the sites of enzymatic activity.

THE PRESENT INVESTIGATION

The material utilized consisted of samples of the placenta of the albino mouse (15th to 18th day of gestation), from that of the albino rat (15th to 18th day of gestation), and from human pla-

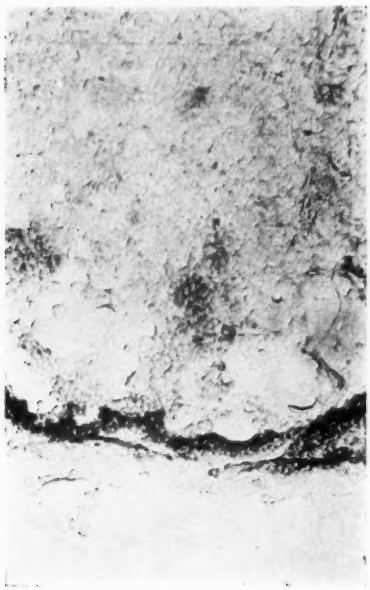

Fig. 1. — Intense succinic dehydrogenase activity in the visceral wall of the yolk sac of mouse. ca. 120 ×.

centa (3rd to 4th month of gestation and full-term). The pieces of the placenta and of the membranes were frozen immediately after removal, sectioned at 50 μ and treated according to the method of Seligman and Rutenberg using neotetrazolium as an indicator for succinic dehydrogenase. The incubation time varied from 30 to 60 minutes. Very thin membranes were also incubated without sectioning.

RESULTS

The Placenta of the Mouse and of the Rat. — There was no essential difference in the succinic dehydrogenase activity of the placenta between these two rodents. The placental labyrinth showed no signs of enzymatic activity. The amnion, both in cross-sections and separately incubated, also remained unstained. The basal decidua revealed a moderate or slight activity by its red color. The site of the highest enzymatic activity was the parietal wall of the yolk sac where the splanchnopleuric villi stained a dark purple by heavy deposition of the small formazan crystals. This precipita-

Fig. 2. — The wall of the yolk sac of mouse incubated separately as a membrane. Vivid activity in the splanchnopleuric villi. ca. 300 ×.

tion of formazan crystals was observed both in cross-sections (Fig. 1) and in the separately-incubated wall of the yolk sac (Fig. 2). The visceral and serosal basement membranes of the vascular splanchnopleure (11) were moderately stained.

Human Placenta. — The succinic dehydrogenase activity of the human placenta, as estimated from the staining intensity, was much weaker than that of the placenta of the mouse and of the rat. No formazan granules could be observed. The staining varied from pale reddish hue to red, indicating sparse enzymatic activity. The amnion was unstained, or, as in some of the specimens, showed a faint reddish hue. The chorion revealed in most cases only a faint reddish color indicating slight succinic dehydrogenase activity. The chorionic villi stained red, thus showing a slight or moderate activity (Fig. 3). The connective tissue of the chorion was unstained. The basal decidua revealed a faint reddish color indicating a sparse enzymatic activity. No substantial differences could be observed in the enzymatic activity of the placentas of the third and fourth months and of those at term.

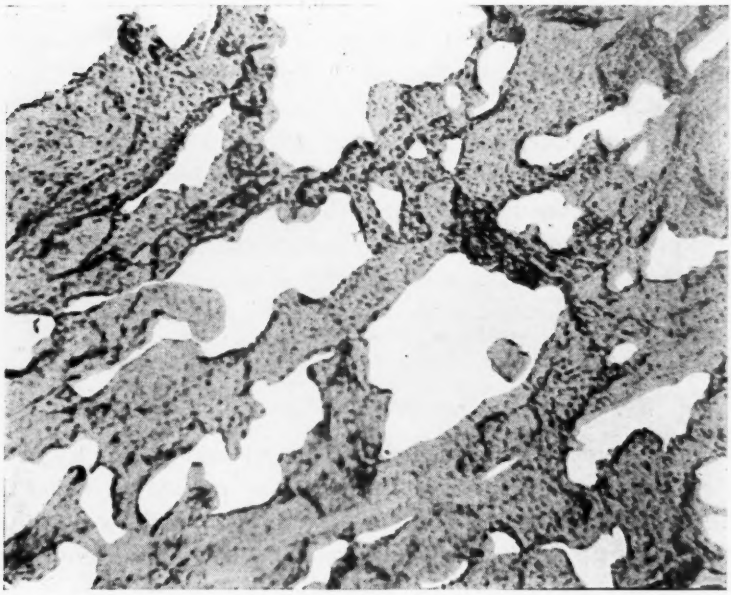

Fig. 3. — Moderate activity in the chorionic villi of the human placenta. ca. 120 ×.

DISCUSSION

Succinic dehydrogenase has been found, both by biochemical (5) and histochemical (4) methods, in greatest abundance in the organs which show high metabolic activity or are engaged in energy-needing absorptive or secretory activities. In these organs the metabolic activity is apparently closely related to the presence of succinic dehydrogenase activity. The intense enzymatic activity in the visceral wall of the yolk sac, vascular splanchnopleure, can be regarded as evidence of its functional activity. In the epithelium of the yolk sac abundantly cytoplasmic granules have been previously observed which have been looked upon by the earlier workers (1, 3) as an indication of a secretory function of the vascular splanchnopleure. In later investigations (8, 11), however, they are believed more likely to be products of absorption. This explanation is confirmed by Everett's (2) observations that the vital dyes are absorbed by the visceral wall of the yolk sac. Our observation on the vivid succinic dehydrogenase activity in the visceral wall of the yolk sac can also be regarded as an indication of its absorptive function

because the most pronounced enzymatic activity has been found in general in the absorptive portions of organs. The moderate activity of the basal decidua of the mouse and rat placenta was almost similar to that of the wall of the uterus.

The great difference in the enzymatic activity between the human and rodent placental tissues can probably be attributable to the fact that the development of the mouse and rat embryos and, consequently, the placental metabolism are more rapid than that of the human embryo. The weak activity of human placental tissues prevents any estimation of its possible significance.

SUMMARY

The histochemically-demonstrable succinic dehydrogenase activity of the placental tissues of the mouse and the rat and of the human placenta and membranes was studied. In the placenta of the rodents the site of the highest enzymatic activity was the visceral splanchnopleuric wall of the yolk sac. The basal decidua showed a moderate activity. The other parts were negative. In human placental tissues the activity was much weaker than in those of rodents. In the chorionic villi a slight activity was noticeable.

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FROM THE DEPARTMENT OF ANATOMY, UNIVERSITY OF HELSINKI, AND FROM THE WIHURI RESEARCH INSTITUTE, HELSINKI

THE RESTORATIVE EFFECT OF CORTISONE ON THE REDUCED ACTIVITY OF SUCCINIC DEHYDROGENASE IN ORGANS OF ADRENALECTOMIZED RAT

A HISTOCHEMICAL STUDY

by

KIMMO K. MUSTAKALLIO, A. N. KUUSISTO and ANTTI TELKKÄ [1]

(Received for publication May 15, 1954)

In a recent histochemical investigation we observed that adrenalectomy was followed by a general reduction in the succinic dehydrogenase activity of rat tissues (4). The present study was carried out in order to investigate whether this decreased enzymatic activity is influenced by the administration of cortisone and desoxycorticosterone.

THE PRESENT INVESTIGATION

45 male albino rats of Sprague-Dawley strain, weighing 125—175 g, were used. Bilateral adrenalectomy was performed on 30 animals, and 15 served as untreated controls. Ten of the animals submitted to operation received 4 mg of cortisone (»Cortone», Merck) intramuscularly daily during seven postoperative days, and 14 mg of a crystal-suspension of desoxycorticosterone acetate (»Percorten», Ciba) was injected intramuscularly to five rats immediately after adrenalectomy. Fifteen of the adrenalectomized rats were not treated with hormones. All the animals were maintained on ordinary mixed food and tap water *ad libitum*. Three of the cortisone-treated animals and their respective untreated and

[1] Aided by a grant from the Niilo Helander Foundation.

adrenalectomized controls were fasted for 24 hours before killing. For the other rats food was available until the sacrifice. Seven days after the adrenalectomy the rats were killed by decapitation. Immediately after killing, pieces from the heart, femoral muscle, kidney, liver, gastric mucosa, pancreas, parotid gland, cerebrum, and cerebellum were treated as described previously (4), following the histochemical method for succinic dehydrogenase introduced by Seligman and Rutenburg (3) using neotetrazolium (NT) as indicator.

RESULTS

In all the organs studied there was, within seven days of adrenalectomy, a general decrease in the succinic dehydrogenase activity as estimated from the diminished staining intensity. This was due to the sparser precipitation of the dark-purple formazan granules. This decline in enzymatic activity was most pronounced in the parietal cells of the gastric mucosa (Fig. 14), in the cells of kidney tubules (Fig. 10), and in the acinar cells of the pancreas (Fig. 17) and the parotid gland.

The administration of cortisone to the adrenalectomized rats restored the succinic dehydrogenase activity to the level of their untreated controls in all the organs studied (Fig. 3, 6, 8, 11, 15, 18, 20). Furthermore, the skeletal muscle, gastric mucosa, kidney tubules, and pancreas of some adrenalectomized rats treated with cortisone revealed even higher activity than their respective controls. The effect of cortisone was most marked when the last dose was administered 2—5 hours before the sacrifice.

Desoxycorticosterone caused no discernible alteration in the reduced succinic dehydrogenase activity of adrenalectomized rats (Fig. 4, 12, 16). The withdrawal of food for 24 hours did not modify either the effect of adrenalectomy or that of cortisone.

DISCUSSION

The observation regarding the general inhibition of succinic dehydrogenase following adrenalectomy confirms our earlier findings (4).

In our experimental conditions cortisone completely restored the decreased succinic dehydrogenase activity called forth by

All the specimens illustrated are NT-preparations of 40 μ.

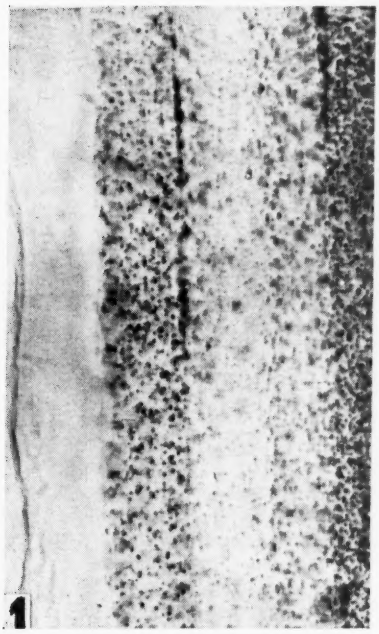

Fig. 1. — Untreated control rat. Femoral muscle. $\times 500$.

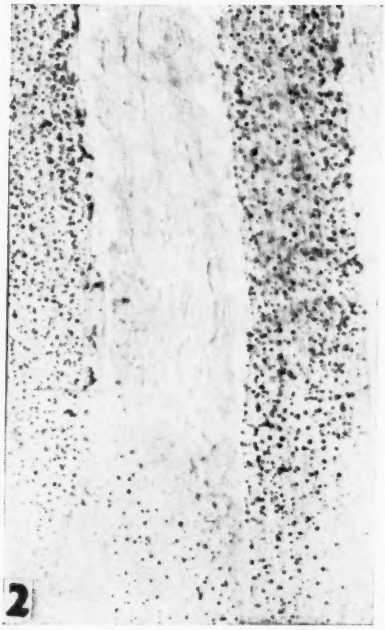

Fig. 2. — Adrenalectomized control rat. Femoral muscle. $\times$ 500.

Fig. 3. — Adrenalectomized rat treated with cortisone. Femoral muscle. $\times 500$.

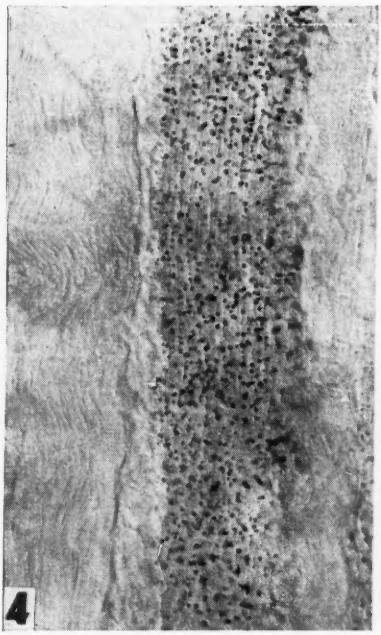

Fig. 4. — Adrenalectomized rat treated with DOCA. Femoral muscle. $\times$ 500.

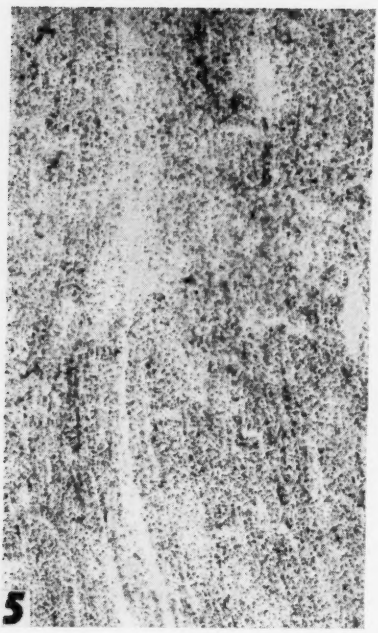

Fig. 5. — Adrenalectomized control rat. Cardiac muscle from left ventricle. × 375.

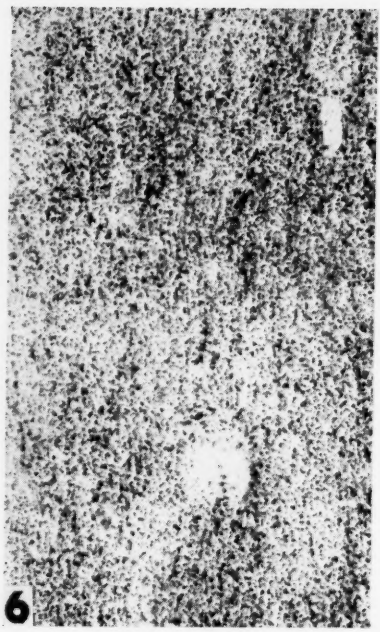

Fig. 6. — Adrenalectomized rat treated with cortisone. Cardiac muscle from left ventricle. × 375.

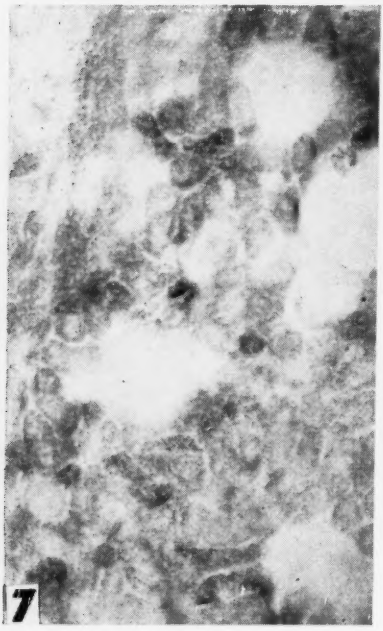

Fig. 7. — Adrenalectomized control rat. Kidney cortex. × 150.

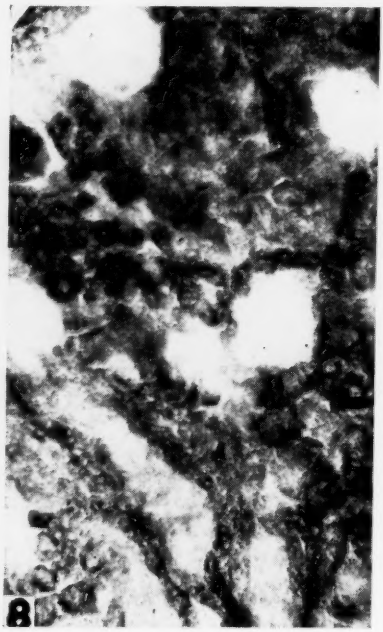

Fig. 8. — Adrenalectomized rat treated with cortisone. Kidney cortex. ×150.

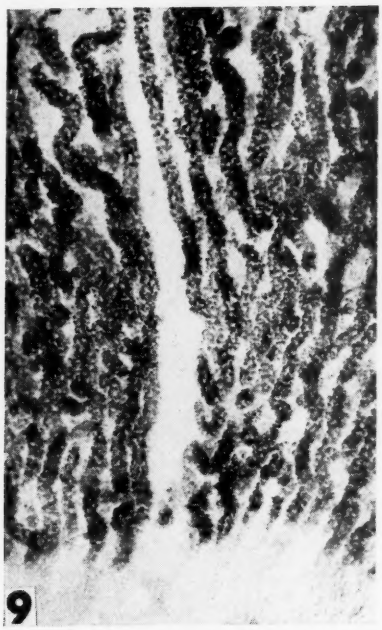

Fig. 9. — Untreated control rat. Thick limbs of Henle's loops × 150.

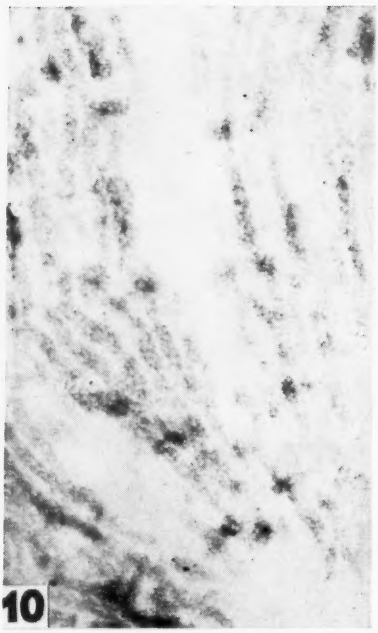

Fig. 10. — Adrenalectomized control rat. Thick limbs of Henle's loops × 150.

Fig. 11. — Adrenalectomized rat treated with cortisone. Thick limbs of Henle's loops × 150.

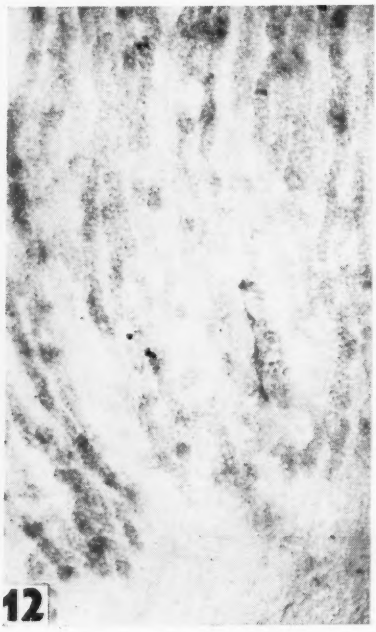

Fig. 12. — Adrenalectomized rat treated with DOCA. Thick limbs of Henle's loops × 150.

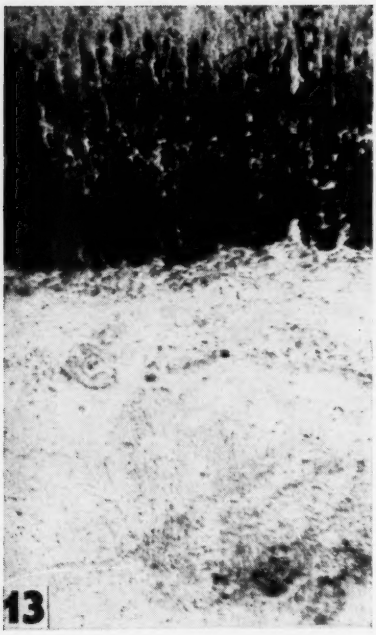

Fig. 13. — Untreated control rat. Gastric mucosa. × 125.

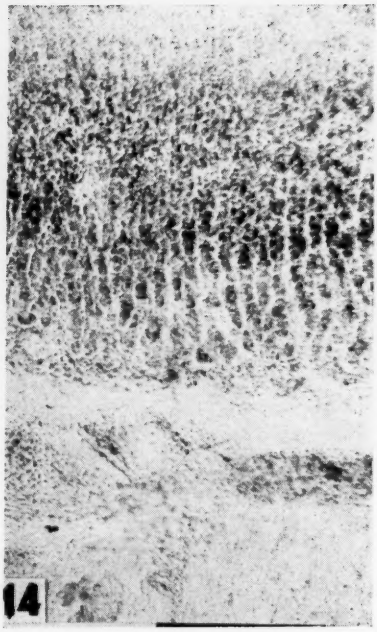

Fig. 14. — Adrenalectomized control rat. Gastric mucosa. × 125.

Fig. 15. — Adrenalectomized rat treated with cortisone. Gastric mucosa. × 125.

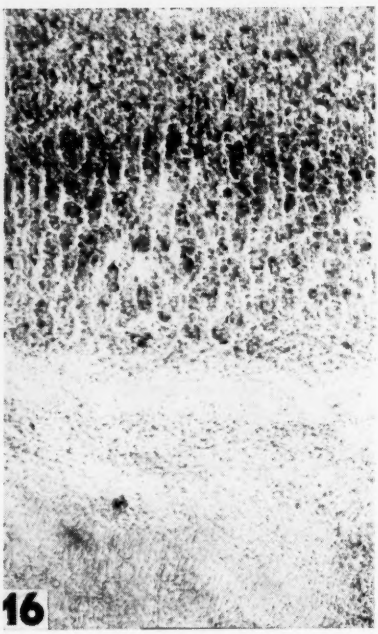

Fig. 16. — Adrenalectomized rat treated with DOCA. Gastric mucosa. × 125.

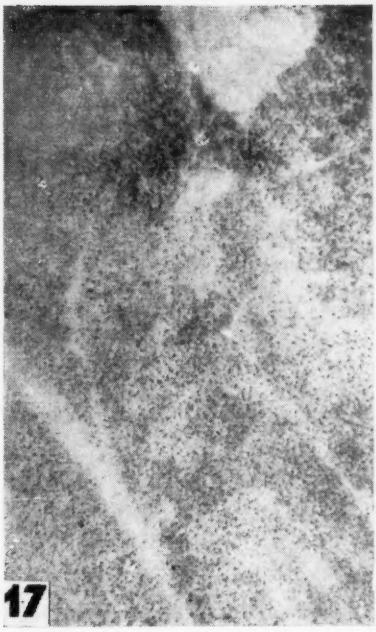

Fig. 17. — Adrenalectomized control rat. Pancreas. × 150.

Fig. 18. — Adrenalectomized rat treated with cortisone. Pancreas. ×150.

Fig. 19. — Adrenalectomized control rat. Corpus striatum. × 300.

Fig. 20. — Adrenalectomized rat treated with cortisone. Corpus striatum × 300.

adrenalectomy, whereas the activity remained unaltered by the administration of desoxycorticosterone acetate.

In the light of the observation made, that cortisone is capable to restore the reduced succinic dehydrogenase activity in the gastric mucosa of the adrenalectomized rat, it is interesting to note that the significant decrease in gastric secretion following adrenalectomy could be corrected with adrenal cortical extract and cortisone (5, 2). Also desoxycorticosterone acetate, in certain conditions, increased the gastric juice volume of adrenalectomized rat (2), whereas in other experiments (5) it was found to be ineffective in this respect.

The observation of Di Bella (1) that the succinic dehydrogenase activity of the rat liver removed one week after adrenalectomy was not altered by the presence of desoxycorticosterone acetate is compatible with our results.

Since cortisone was found to be effective in restoring the depressed succinic dehydrogenase activity following adrenalectomy, it seems probable that of adrenal hormones the glucocorticoids play the most important role in maintaining the normal succinic dehydrogenase activity of the tissues.

SUMMARY

In adrenalectomized rats the reduced succinic dehydrogenase activity of tissues was completely restored by the administration of cortisone, whereas desoxycorticosterone was ineffective in this respect.

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FROM THE DEPARTMENT OF PHYSIOLOGY, UNIVERSITY OF HELSINKI

THE STRUCTURE OF LIVING NERVE FIBRE

FURTHER STUDIES WITH THE ANOPTRAL CONTRAST MICROSCOPE

by

MAIJA-LIISA KOTILAINEN and ALVAR WILSKA

(Received for publication May 15, 1954)

In a previous publication (1) we gave a few preliminary notes on the subject. This investigation also deals with the myelinated nerve fibres only. According to all the textbooks such fibers are composed of an optically empty axis cylinder (axon) surrounded by the myelin sheath. The continuity of this sheath is disturbed at the nodes of Ranvier where the sheath almost vanishes. The sheath is also interrupted by the Schmitt-Lantermann incisures, funnel-like clefts which open irregularly in either direction. With special staining methods granules and fibrils have been demonstrated in the axoplasm. Some investigators even claim to have seen tubular formations (neurotubuli) within the axon. Treatment of the myelin sheath with lipid solvents leaves a network which is believed to consist of keratin.

A very detailed electron-microscopic analysis of the nerve fibre structure has been made by Fernández-Morán (2). According to him the myelin sheath is made of concentric lamellae. Between these myelin layers irregular clefts can be seen. There is a network of fine fibrils in the axoplasm. In the meshes of this network are located vesicles of different size. The network is denser and more uniform at the outer border of the axoplasm, thus forming the axolemma. Fernández-Morán has also succeeded in making excellent electron-micrographs of fragments of myelin torn from the axon sheath. These pictures show molecular myelin layers super-

imposed on one another which sometimes show up in staircase-like formations with steps only 80 Ångström units in height. As Fernández-Morán has pointed out, the structure of fresh living axoplasm remains invisible when examined under ordinary bright field, dark field, phase contrast, polarization and ultra-violet microscopes.

Using the anoptral contrast method, the name given to the new microscopic contrast principle (3—5) by its commercial producer, Reichertwerke of Vienna, we have examined nerve fibers of both mice and frogs. The similarity of the fibers in both these remote species of animals is striking. Individual fibers from a short piece of the sciatic nerve lying in Ringer solution were separated from one another with fine needles and a microscopic specimen was made, care being taken to avoid compression by the coverglass. According to the earlier electrophysiological experience of one of us an operation like this does not as a rule abolish the conduction of impulses in the isolated fibre.

Under the anoptral microscope globoid and ovoid vesicular bodies can be seen in the axoplasm. Their size varies between 0.3—3 μ and their total volume constitutes about 1—3 per cent of the volume of the axoplasm. Some of the bodies are more and the others less refractile than their surroundings. In some instances these vesicles seem to be forming chains as though they were varicosities of subtle tubules.

The axoplasm has a jelly-like consistency, as already noted by de Renyi (6) who made micromanipulation experiments on the axon. We have found that the axoplasm is easily extruded or pulled out from the cut end of a fibre without undergoing any noticeable change in its structure. The axoplasm can be stretched to several times its original length without breaking. In its stretched state the vesicles become correspondingly elongated.

The myelin is strongly refractile. Thus the myelin sheath appears in our microscope about 100 times brighter than the axoplasm. A photograph of the myelin structure requires a considerably shorter exposure than for axoplasm pictures. In Fig. 1 we can see how the lamellae in the myelin sheath split after sharp bends or other traumata, just like the layers of a pasteboard tube when damaged.

Staircase formations of myelin like those observed by Fernández-Morán with electron optics can also be seen with the anoptral

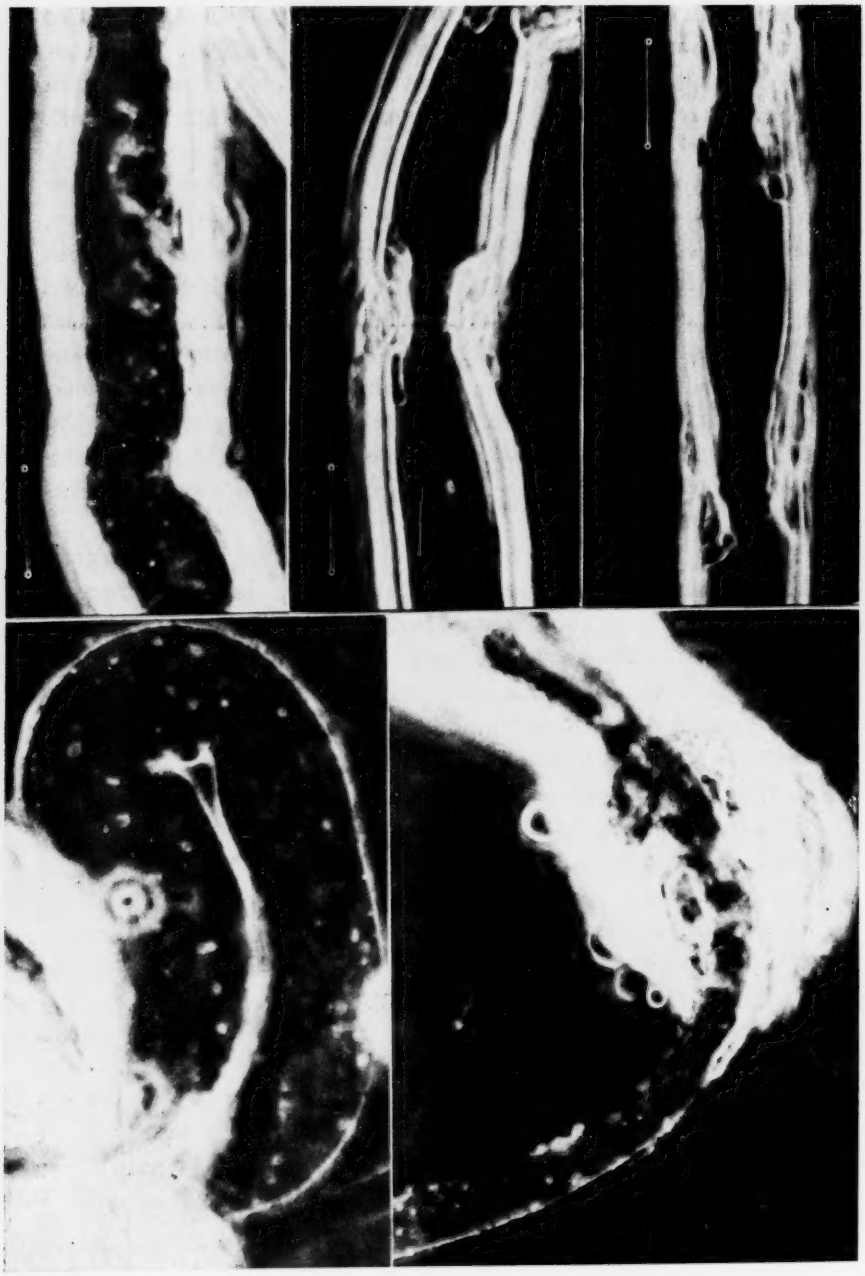

Fig. 1.

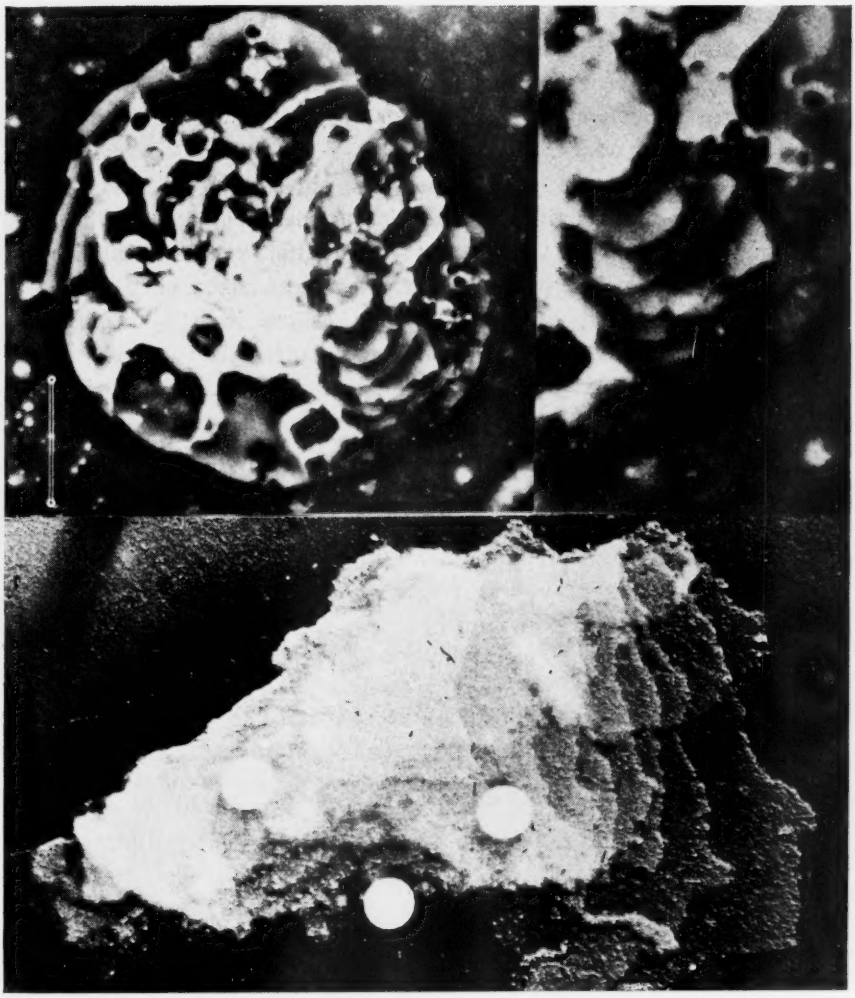

Fig. 2. — Above: A small piece of myelin torn off the axon sheath and photographed with the anoptral contrast (Reichert 100× oil-immersion). Magnification: left 1600, right 3000. Below: A similar pile of 8 myelin lamellae, electron-optically photographed by Fernández-Morán. Dow-Latex calibration spherules have a diameter of 2600 Å.

◀ Fig. 1. — Above: Axons from the sciatic nerve of a rat. The picture to the left shows an undamaged axon in which the axoplasm structure is visible. The myelin sheath is overexposed. In the second and third picture with slightly damaged axons the exposure has been much shorter. The myelin, with its splitting into laminae at damaged points, is more clearly visible. Below: Axoplasm extruded (left) and pulled out (right) from the cut end of an axon. Vesicular bodies are clearly visible. Magnification 1400. Reichert anoptral 100× oil-immersion.

(Fig. 2). The steps can be seen very clearly although, according to Fernández-Morán, they are only 80 Ångström units thick. This is no wonder, however, since our microscope shows even considerably thinner membranes made by dropping collodion-amylacetate solution on a water surface.

Our observations do not support the old conception that the Ranvier nodes are genuine and essential formations of a nerve fibre. The more carefully the preparation is made, the fewer the Ranvier nodes that can be observed in it. By careless treatment we can create almost any amount of formations which are indistinguishable from the »real» nodes of Ranvier. We do not deny the possibility of the existence of these nodes in living nerves in situ, but even they may have been provoked by some local traumata during the life of the animal in question. We feel there is every reason for the histologist to reinvestigate and reconsider the question of these nodes.

We have almost never seen Schmitt-Lantermann incisures in carefully made fresh preparations. Osmic acid appears to be especially effective in provoking these formations into being. That these conical clefts open now in one direction, now in another speaks for their exogenous origin.

SUMMARY

A study of living nerve fibers with the anoptral contrast microscope shows that many electron-microscopically observed structures such as vesicles in the axoplasm and myelin lamellae can also be seen under living conditions. Anoptral observations cast some doubt upon the genuineness of Ranvier nodes and especially of Schmitt-Lantermann incisures.

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FROM THE SECOND WOMEN'S CLINIC AND FROM THE DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF HELSINKI

URINARY EXCRETION OF 17-KETOSTEROIDS IN HYPEREMESIS GRAVIDARUM

by

VEIKKO J. UUSPÄÄ[1] and PENTTI A. JÄRVINEN

(Received for publication May 18, 1954)

Urinary 17-ketosteroids are in females almost wholly derived from the metabolism of adrenocortical, in males also of testicular hormones. The average excretion of 17-ketosteroids ranges in normal women from 6.8—13.2 mg/24 hrs (7, 21, 22, 28). The destruction or atrophy of the adrenal cortex causes an almost complete loss of urinary 17-ketosteroids in females, the excretion falling, occasionally, even below 1.0 mg/24 hrs (3, 21, 30). Hyperfunction of the adrenal cortex, on the other hand, is reflected by increased excretion of 11-oxycorticosteroids and frequently also of 17-ketosteroids, though no parallelism between the urinary excretion of 17-ketosteroids and 11-oxysteroids has been observed (6, 30, 19). The injection of ACTH is most frequently followed by increased excretion of 17-ketosteroids. Here, however, we have to take into consideration the possibility that 17-ketosteroids may increase as a result of the degradation of the 11-oxysteroids (27)

The pituitary gland and adrenal cortex have a tendency to increased activity during pregnancy. Therefore, it is to be expected that the excretion of 17-ketosteroids should also increase during pregnancy. Jailer (16), as a matter of fact, has observed an increase in the excretion of 17-ketosteroids since the 3rd to 5th months of pregnancy, though to a lesser degree than in excretion of cortico-

[1] Fellow, Rockefeller Foundation.

steroids. Caton et al. (4) and others (1, 2, 26, 13) have obtained somewhat similar results. However, marked alterations in the excretion of 17-ketosteroids during pregnancy have not been found in various other investigations (10, 9, 5, 24, 12). Venning (29) has noted that the values of 17-ketosteroids obtained with the Pincus method (23) did not alter during pregnancy, while the Holtorff-Koch method (14) revealed increased values for excretion of ketosteroids during pregnancy. Venning is of the opinion that the excretion of 20-ketosteroids is increased during pregnancy, contrary to that of 17-ketosteroids. Hegnauer (12) recently noted a decrease in the urinary excretion of 17-ketosteroids in five patients with hyperemesis. Prosdocimi and Giaquinto (25) have reported that in ten patients with hyperemesis the urinary excretion of 17-ketosteroids ranged from 3.6 to 6.08 mg/24 hrs, with an average of 4.8 mg/24 hrs.

In a recent paper (18) results suggesting hypofunction of the adrenal cortex have been obtained with Thorn's test in cases of hyperemesis gravidarum. The purpose of the present work has been to find out whether the insufficiency also concerns the part of adrenal cortex which produces androgens.

METHODS

Since 1951, 17-ketosteroid determinations were carried out in 32 patients with hyperemesis gravidarum and in 22 normal pregnant women during the first trimester of pregnancy. The urine specimens were collected during two subsequent days after the admission, before treatment was instituted. Altogether, 104 determinations of 17-ketosteroids were carried out according to the Jensen and Tötterman method (17), by using Zimmermann's (31) reagent. The values obtained were corrected by means of the equation of Gibson and Evelyn (8).

RESULTS AND DISCUSSION

The excretion of 17-ketosteroids in patients with hyperemesis gravidarum ranged from 0.5 to 11.6 mg/24 hrs, with an average of 5.3 mg/24 hrs. The corresponding values in normal pregnant women were 2.4 to 15.7 mg/24 hrs and 8.1 mg/24 hrs (Table 1).

TABLE 1

	No. of Cases	Average Age (Years)	Excretion of 17-ketosteroids	
			Variation Limits mg/24 hrs	Average
Normal Pregnancy	22	30.4	2.4—15.7	8.1
Hyperemesis gravidarum	32	29.3	0.5—11.6	5.3

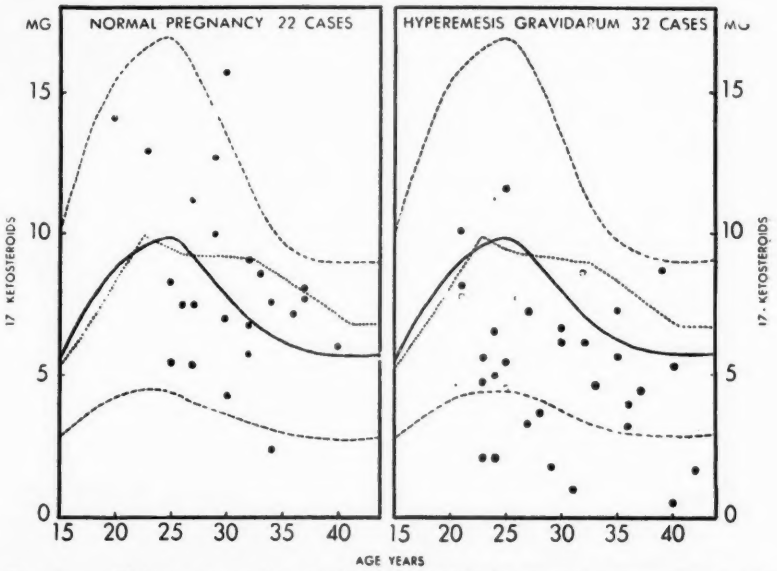

Fig. 1. — Urinary excretion of 17-ketosteroids in normal pregnancy and in hyperemesis gravidarum. Dots illustrate the average excretion (mg/24 hrs) during two subsequent days (48 hrs). O = one determination only has been performed. Unbroken line presents the normal average curve of 17-ketosteroids in non-pregnant women of various ages according to Hamburger (11). Dashed lines show the variation limits. Dotted line presents average urinary excretion of 17-ketosteroids (mg/24 hrs) in non-pregnant Finnish women of various ages according to Pesonen (22).

The comparison of the present values with those observed by Hamburger (11) and Pesonen (22) in non-pregnant women is illustrated by the graph in Fig. 1.

During the first trimester of pregnancy the excretion of 17-ketosteroids seems to be approximately at the same level in normal pregnant women and in non-pregnant women of the same age. The mean value of the diurnal excretion (8.1 mg) was almost the same as that observed by Pesonen with the same method (8.6 mg) in non-pregnant women of the age group 21 to 42 years.

The excretion of 17-ketosteroids in patients with hyperemesis gravidarum was on an average somewhat smaller than in normal pregnant women. In ten out of the 32 patients of hyperemesis series the excretion did not exceed Hamburger's minimum curve, in the control group it fell below it in one subject only. However, in three patients with hyperemesis gravidarum and in seven subjects in the control group the excretion of 17-ketosteroids was greater than the average values observed by Pesonen. The decrease of the excretion in hyperemesis series may be partly due to inanition, in which Jacobs (15) and Landau *et al.* (20) observed a decrease of 50 per cent in the excretion of 17-ketosteroids. Since, however, in most patients with hyperemesis gravidarum the excretion was considerably lower than in normal non-pregnant women, in six patients even below 2.5 mg/24 hrs, it may be reasonable to assume that the stimulus which controls the androgen production of the adrenal cortex has been temporarily inhibited. The results seem to support the assumption that hyperemesis gravidarum is due to temporary hypofunction of several adaptation systems.

SUMMARY

To elucidate the function of the adrenal cortex in hyperemesis gravidarum the urinary excretion of 17-ketosteroids was determined in 32 patients with hyperemesis and in 22 normal pregnant women during the first trimester of pregnancy. The excretion of 17-ketosteroids ranged from 0.5 to 11.6 mg/24 hrs, with an average of 5.3 mg/24 hrs in patients with hyperemesis gravidarum, and from 2.4 to 15.7 mg/24 hrs, with an average of 8.1 mg/24 hrs in normal pregnant women. The results seem to suggest that the function of adrenal cortex is impaired in hyperemesis gravidarum.

The writers are indebted to S. Pesonen, Ph. D., under whose guidance the determinations of 17-ketosteroids were performed.

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FROM THE FIRST SURGICAL UNIVERSITY CLINIC AND FROM THE UNIVERSITY DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, HELSINKI

BACTERIAL AND FUNGAL FLORA OF BRONCHIAL SECRETION IN DISEASES OF THE CHEST

WITH SPECIAL REFERENCE TO ANTIBIOTIC THERAPY

by

MARTTI TURUNEN, KARI ASP, and E. A. HELLAAKOSKI

(Received for publication June 17, 1954.)

There are several routine aids available for the diagnosis and treatment of diseasis of the chest. Most of the published studies of the flora of the respiratory tract deal with an analysis of sputum and pharyngeal secretions, while bronchial secretion seems to have received less attention, notwithstanding the fact that it is produced nearer the diseased tissue and can therefore be assumed to give a truer notion of the state of the lung than does sputum. As far as we know, there is no extensive study of changes produced by antibiotic drugs in the number of micro-organisms and their mutual proportions, nor of their resistance to antibiotic therapy, based on bronchoscopically-obtained samples of bronchial secretion.

THE PRESENT INVESTIGATION

Material and Technique. — The series consisted of 48 cases treated in the First Surgical University Clinic, Helsinki. Altogether 53 samples of bronchial secretion were taken. In 15 cases a sputum sample was taken as well. The distribution of the various pathological conditions within the series was as follows: bronchiectasis, 21 cases; pulmonary tumours, 9; pulmonary tuberculosis, 6; lung lesion caused by a foreign body, 3; pulmonary abscess, 2; other

pulmonary conditions, 7. In 5 cases of bronchiectasis, two samples were taken from each patient.

The samples of bronchial secretion were taken either by aspiration at bronchoscopy or by washing the bronchus with sterile saline. Immediately after the obtaining of the sample, a slide preparation was made and stained by Gram's method; in addition there were bacterial cultures inoculated on blood plates, Drigalski—Condradi, and McLeod agars and in thiogluconate or liver broth tubes. Tbc culture was carried out by the Kirchner method and the fungus culture in thiogluconate, Saboraud's milieu d'épreuve, and oil medium (Gordon 1951), and in a tube with nutrient medium used for special culture (Penttinen and Rauramo 1948).[1] Bacterial cultures from the sputum were carried out in the same way.

RESULTS

A. Occurrence of Bacteria and Fungi in Bronchial Secretion. — An analysis of this is summarised in table 1.

The table shows that the occurrence of bacteria in bronchial secretion varied greatly. The predominant kind is a non-defined *Gram-negative rod*, of the influenza type. Several investigators (Mulder, Goslings, van der Plas, and Lopes Cardozo 1952) have called attention to the occurrence of these rods. They believe that the *H. influenzae* is of a decisive pathological significance in all kinds of infections of the respiratory tract. *Neisseria* was present in 9 cases, *Micrococcus saprophyticus* in 8, *Streptococcus α-haemolyticus* in 6, and *Pneumococcus* in 5 cases. Other bacteria were present in one or two cases. Culture revealed no tbc bacilli in any of the samples. The occurrence of the various bacteria was, in general, evenly distributed between the different diseases. This is in agreement with the observation of Isaerson and Neelova (1948), that there is no parallelism between the clinical features and the bacterial flora of the sputum.

It seems rather striking to us that *Proteus vulgaris* was present in two cases of suppuration due to a foreign body. Both patients were wounded in the chest. It is possible that the presence of

[1] We take this opportunity of expressing our sincere thanks to Dr. Risto Pätiälä. We have greatly benefited by his expert knowledge in matters relating to fungi.

TABLE 1

RESULTS OF BACTERIAL AND FUNGAL CULTURES GROUPED BY DISEASES

	Bronchiectasis	Tumour	Tuberculosis	Foreign Body	Pulmonary Abscess	Other Diseases	Total
Gram-negative rods ..	6		2	1		2	11
Neisseria	6	2		1			9
Micrococcus saprophyticus	2	3	1		1	1	8
Str. *α*-haemol.	3	1	1	1			6
Pneumococcus	2	2				1	5
Proteus vulg.				2			2
Micrococcus pyog. v. aureus	1						1
Micrococcus pyog. v. albus	1						1
Chromobact. flavi	1						1
Gaffkya			1				1
Diphteroid	2						2
E. coli	1						1
Fæcalis alk.	1					1	2
Sarcina lutea		1					1
B. fusiformis	1		1				2
Neg. bact. culture	11	2	1	1	1	3	19
Penicillium	6	3				1	10
Yeast	2	1			1		4
Fucarium	1						1
Geotrichum	1						1
Actinomyces				1			1
Neg. fungal culture ..	19	5	6	2	1	6	39
Total No. of patients	21	9	6	3	2	7	48
Total No. of samples ..	26	9	6	3	2	7	53

Proteus is characteristic of foreign body suppuration; this assumption is supported by its frequent occurrence in infections caused by renal calculi. Another noteworthy fact was that *Micrococcus pyogenes v. aureus* was present only in one case; it may or

may not have invaded the lung at bronchography carried out the preceding day.

In 19 cases bacterial culture yielded a negative result. In some of these cases, native preparations exhibited some *Gram-negative rods*. In all the cases where bacterial culture was positive, the native preparations contained the bacteria.

The native preparations and the results of bacterial culture as compared with each other:

Bacteria recovered in native preparations ..	42	samples
Bacteria recovered by cultivation	34	»
Negative native preparations and cultures ..	11	»

Eight samples revealed bacteria which did not grow on culture media. All these bacteria were *Gram-negative rods*, evidently of the influenza type, which may account for their failure to grow on culture media. The cases in which the native finding was positive but the cultivation negative were evenly distributed between the different diseases.

In order to estimate the abundance of the bacteria, their number was computed from native preparations within a field of vision chosen at random. Table 2 shows the sums of 5 fields of vision from each preparation, grouped according to the diagnoses.

TABLE 2

OCCURRENCE OF BACTERIA WITHIN 5 FIELDS OF VISION OF THE MICROSCOPE

	No Bacteria	0—20 Bacteria	More than 20 Bacteria
Bronchiectasis	6	12	8
Tumour	1	4	4
Tuberculosis	1	2	3
Other diseases	3	5	4
Total	11	23	19

Patients with tumour had a considerable number of bacteria. The other diseases show a more even distribution.

Table 1 shows that in 14 cases fungi were cultivated from the bronchial secretion, i.e., in 26 per cent of the whole series. The positive cultures were fairly evenly distributed between the different diagnosis groups except that the cases of tuberculosis showed no

positive growth. Not very much is known about the occurrence of fungi in the respiratory tract, but they are fairly frequently present in the mouth and the pharynx. The results of our study are in rough agreement with those dealing with the occurrence of fungi in the mouth and the pharynx (Todd 1937, Epstein, Lampert, Haler, etc.).

B. The Effect of Antibiotic Therapy on the Occurrence of Bacteria and Fungi. — The patients were treated mainly with procaine penicillin and streptomycin. Other antibiotics were seldom used and the dosages were small. The patients received either penicillin alone or penicillin and streptomycin simultaneously. The series included no case treated by streptomycin alone. The usual daily dosage was 600,000 units of procaine penicillin and 0.5—1.0 g of streptomycin. The drug was injected intramuscularly.

Immediately before the sample was taken 23 patients received antibiotic therapy. A total of 26 samples were taken from these patients. There were 25 untreated patients, from whom a total of 27 samples were taken. Both groups were thus of equal size.

TABLE 3

THE OCCURRENCE OF BACTERIA AND FUNGI IN CASES NOT TREATED BY ANTIBIOTIC DRUGS, GROUPED ACCORDING TO THE DIAGNOSES

	No Bacteria	Native Preparation Positive	Cultivation Positive	Fungal Cultivation Positive
Bronchiectasis	2	11	8	2
Tumour	1	3	2	2
Tuberculosis	1	1	1	—
Other diseases	2	5	5	6
Total	6	20	16	2

TABLE 4

THE OCCURRENCE OF BACTERIA AND FUNGI IN CASES TREATED BY ANTIBIOTIC DRUGS, GROUPED ACCORDING TO THE DIAGNOSES

	No Bacteria	Native Preparation Positive	Culture Positive	Fungal Culture Positive
Bronchiectasis	4	8	7	2
Tumour	—	5	5	2
Tuberculosis	—	4	4	—
Other Diseases	1	4	2	1
Total	5	21	18	5

Tables 3 and 4 show that penicillin and streptomycin therapy had no particular effect on the bacteria and fungi of bronchial secretion, for the results in the treated and untreated series were roughly the same. The same can be said of the occurrence of individual bacterial and fungal strains in the two series, for each microorganism was equally distributed in both series. Haffner, Neter, and Rubin (1950), who studied the bacterial flora of the pharynx, found that the number of Gram-positive organisms lessened during penicillin treatment and that Gram-negative bacteria became predominant. We did not make the same observation because it seems that a Gram-negative flora predominates in bronchial secretion even before the administration of antibiotics.

Neither were penicillin nor streptomycin in the doses used found to have any effect on the fungal flora. The action of antibiotic drugs on fungi has not been fully elucidated. Campbell and Saslaw (1949) found that streptomycin promoted the growth of certain fungal strains *in vitro*, while Zahawi and Semah (1951) and Malamud and Stepankowsky (1951), for example, have obtained good results in pulmonary moniliasis by administering streptomycin alone or in combination with other drugs. Lipnik, Kligman, and Strauss (1952), who studied the effect of various antibiotic drugs on the fungal flora of the mouth and rectum, found penicillin therapy pretty inefficient.

The poor results obtained by antibiotic therapy might possibly be due to too small dosage. The greatest total doses now used are about 20 million units of penicillin and about 15 g of streptomycin. The majority of the patients in the present series received less than 10 million units of penicillin and less than 10 g of streptomycin. The main reason for the poor result, however, seems to be the poor secretion of the antibiotics into the bronchial secretion. In view of this, attempts have been made to produce a penicillin preparation which would be secreted through the lungs. A preparation of this type is Leocillin (Leo). Inhalators of various kinds have also been used to secure for the antibiotics a straight access to the bronchial mucosa. Pätiälä and Savolainen (1951), who studied the secretion of procaine penicillin and Leocillin into sputum, found that the concentration of Leocillin was four or five times that of procaine penicillin when equal dosages were used. Leocillin was not used in the treatment of the patients in the present series because

TABLE 5

RESISTANCE OF DIFFERENT BACTERIA

	0		1		2		3		4		5	
	Penicillin	Streptomycin	Penicillin	Streptomycin	Penicillin	Streptomycin	Penicillin	Streptomycin	Penicillin	Streptomycin	Penicillin	Streptomycin
Gram-negative rods..	4	5					1					
Neisseria		2		1			3	1	1			
Micrococcus saprophyticus		1			1	3	2	1	2			
Str. α-haemol.			1			1	2	2				
Pneumococcus			1			2			3			
Proteus vulgaris	2	2										
Micrococcus pyog. v. aureus							1					
Micrococcus pyog. v. albus		1										
Chromobact. flavi ..		1										
Gaffkya												
Diphteroid	1	1										
E. coli			1							1		
Fæcalis alkal.	1	1										
Sarcina lutea												
B. fusiformis												

The sensitivity is rated 0—5, 5 indicating the greatest degree of sensitivity.

during an earlier application of Leocillin in our clinic three nurses contracted skin allergy.

Bacterial resistance in the bronchial secretion also suggested poor secretion of penicillin and streptomycin, as seen from table 5.

C. The Sensitivity of Bacteria in Bronchial Secretion to Penicillin and Streptomycin. — The sensitivity of all cultivated bacteria was tested by the plate method. Thirty-four positive cultures yielded a satisfactory growth on 15 test plates only. The sensitivity was rated 1 to 5. The results are presented in table 5.

Table 5 shows that the *Micrococcus saprophyticus, Streptococcus hæmolyticus,* and *Pneumococcus* were comparatively sensitive to

penicillin and streptomycin; yet they were present in cases treated by these antibiotics. The same observation was made by Dulfano and Segal (1953), who studied the effect of Neo-Penil on the bacterial flora of sputum. They found that the *Streptococcus α-hæmolyticus*, *Neisseria flavus*, and *Neisseria catarrhalis* were present after treatment as well as before it. They also noted that in some cases these organisms emerged in the sputum even during treatment. We made a similar observation in 3 cases in which two samples were taken at certain intervals.

All these patients suffered from bronchiectasis. None of them showed bacteria or fungi on the first cultivation. For a month the patient received procaine penicillin and streptomycin. In another case, *Neisseria* and *penicillium* were cultivated from a sample of bronchial secretion. From another sample, taken 5 months later, when the patient had been treated by 5 million units of penicillin and terramycin, *Neisseria*, *Micrococcus saprophyticus*, and the *yeast fungus* were cultivated. In a third case, *Streptococcus α-hæmolyticus* and *Neisseria* were cultivated before antibiotic treatment. Immediately before the taking of the following sample the patient was administered penicillin and streptomycin for 5 days. Culture yielded a penicillin-sensitive *Micrococcus pyog. v. aureus*. On the preceding day the patient had been submitted to bronchography.

D. The Leukocyte Count of Bronchial Secretion. — An attempt was also made to count the number of leukocytes in the bronchial secretion, though counting their number per cu mm proved to be a matter of some difficulty. In many cases the sample had been taken by washing the bronchus with saline, and in a number of cases the sample was so small that it was not possible to count the leukocytes in the usual way. Therefore, to get a rough idea, of the number of leukocytes in the bronchial secretion, we counted them from a stained native preparation within 5 arbitrarily chosen fields of vision. The results, which showed considerable variation, are presented in table 6. The leukocyte count was carried out from a total or 43 samples.

It is worthy of note than there were only two cases of bronchiectasis with remarkable leukocytosis. This may be due to chronic suppuration, which must have weakened the patient's leukocyte response. The group of »other diseases» is strongly represented in the column of slight leukocytosis, which is accounted for by the

TABLE 6

THE OCCURRENCE OF LEUKOCYTES IN 5 MICROSCOPIC FIELDS

	1—10 Leukocytes	11—20 Leukocytes	More than 20 Leukocytes
Bronchiectasis	3	12	2
Tumour	3	2	4
Tuberculosis	1	2	3
Other diseases	6	2	3
Total	13	18	12

TABLE 7

BACTERIAL FLORA IN BRONCHIAL SECRETION AND SPUTUM

Bronchial Secretion	Sputum
1. Negative culture	Pneumococcus
2. » »	E. coli, Streptococcus α-hæmolyticus, Fæcalis alcaligenes
3. » »	Streptococcus α-hæmolyticus, Neisseria
4. » »	Streptococcus α-hæmolyticus, Pneumococcus
5. Gaffkya, Gram-negative rods	Gram-negative rods, Streptococcus α- and β-hæmolyticus
6. Neisseria, Streptococcus α-hæmolyticus	Neisseria, Streptococcus α-hæmolyticus
7. Proteus vulgaris, Gram-negative rods	Neisseria, Streptococcus α- and β-hæmolyticus
8. Proteus vulgaris	Proteus vulgaris, Klebsiella, H. influenzæ
9. Pneumococcus, Micrococcus saprophyticus	Micrococcus pyog. v. aureus
10. Micrococcus saprophyticus	Streptococcus α- and β-hæmolyticus, Neisseria
11. Streptococcus α-hæmolyticus, Fæcalis alkaligenes	Neisseria, Gram-negative rods
12. Neisseria, Gram-negative rods	Neisseria, Pneumococcus
13. Neisseria	Neisseria, Streptococcus α-hæmolyticus, H. influenzæ
14. Pneumococcus	E. coli, Streptococcus α-hæmolyticus, Neisseria
15. Micrococcus saprophyticus	Neisseria, Streptococcus α-hæmolyticus

fact that these diseases were not highly inflammatory (chondroma of the chest wall, a foreign body in the lung, infracardiac cyst, esophageal fistula, etc.). The smallness of the series and the less accurate method of counting the leukocytes render any definite conclusions impossible.

E. The Bacterial Flora of Bronchial Secretion Compared to That of Sputum. — In 15 cases samples were taken simultaneously from the bronchial secretion and sputum. The different diseases were represented as follows: bronchiectasis, 2 cases; tumour of the lung, 7; foreign body in the lung, 2; pulmonary asthma, 1 case; tuberculoma and a foreign body in the thoracic wall, 1. Table 7 records the results of culture in each case.

Table 7 shows that bacterial growth was much richer from sputum than from bronchial secretion. It is also worthy of note that bronchial secretion and sputum showed the same bacteria in 5 cases only. This shows that bronchial secretion is a more reliable indicator of the real state of the lungs. The same conclusion was arrived at by Isserson and Neelova (1948) and Schinz (1952).

DISCUSSION

It is obvious from our study that the flora of bronchial secretion is remarkably rich. There was nothing in the series studied to support the observation made by Angela and Barbanti (1950), that the bacterial flora of pus originating from the diseased part of the lung has a bearing on the type of suppuration present. These authorities found that in cases of an acute abscess the pus contained hemolytic streptococci and that in bronchiectasis, residual cavities, and cysts it contained staphylococci; in cases of diffuse suppuration the bacterial flora varied. One reason for the qualitative richness of the bacterial flora may have been the fact that nearly all the patients in the present series had a severe organic disease which presumably had brought about an extensive alteration in the condition of the bronchi. An adequate control series would, of course, have clarified the matter, but it was impossible to have a control series since it was extremely difficult to persuade normal subjects to submit themselves to bronchoscopy. The series, however, included 4 cases in which the conditions approximated normal ones. These cases were those of chondroma of the chest wall, esophageal

diverticulum, a penetrating wound of the chest, and infracardiac cyst. Culture revealed the following organisms in these cases: (1) *Pneumococcus* and *Micrococcus saprophyticus*, but no fungi, (2) *Gram-negative rods* and *Penicillium;* (3—4) here the bacterial and fungal cultures were negative. These four cases suggested, thus, that the bronchi do have a bacterial flora of their own, but the smallness of this control group forbids any conclusions.

The fungal flora was no less variegated than the bacterial flora. It was not possible to discern any definite groupings in it except that fungal infection was not present in any of the cases of tuberculosis.

It has often been found that fungi present in the upper parts of the respiratory tract may produce true pulmonary mycoses. In Finland this problem has been studied by Elo, Pätiälä, and Ritama (1951), who succeeded to cultivate *Actinomyces bovis* in two cases and *Candida albicans* in one case from a bronchoscopically-obtained sample. The pathological features of the disease were incontestably those of pulmonary mycosis. None of the cases in our series revealed features suggestive of pulmonary mycosis, which suggests that saprophytic fungi discovered in the mouth and pharynx may also be present as saprophytes in the bronchi. Hall (1951) believes that moniliasis of the respiratory tract is caused by vitamin B deficiency. He reported 6 cases which gave a favourable response to large doses of vitamin B.

While we did not note any effect of penicillin and streptomycin on the bronchial flora, chloromycetin and aureomycin, in particular, have been reported to promote the growth of fungi. Meads, Rove, and Haslam (1951) discovered, after aureomycin treatment, fungi in the mouths of patients who previously did not show any. Lipnik, Kligman, and Strauss (1952) noticed that aureomycin therapy increased the fungal flora of the mouth and rectum. Woods, Manning, and Patterson (1951) believe that antibiotic drugs, by destroying the bacterial flora, give the fungal flora better possibilities of nutrition.

The results of our comparison between the bacterial flora of the bronchial secretion and that of sputum speak against the reliability sputum examinations in determining the presence and resistance of bacteria in various pulmonary processes. On the other hand, a sample of bronchial secretion is considerably more difficult

to obtain, and it, too, may give a highly varying notion of its bacterial flora, even within one and the same case, so too much significance ought not to be attached to it. Leukocytosis in the bronchial secretion may deserve careful attention because the number of leukocytes may give an idea of the resistance power of the human organism and of bacterial virulence, two factors which are of decisive importance for the course of the infection.

SUMMARY

A total of 53 samples of bronchial secretion taken bronchoscopically from 48 pulmonary patients were examined for bacteria. The bacterial flora was comparatively rich and apparently independent of the quality of the pathological process. The most common kind of bacterium was a *Gram-negative rod. Proteus vulgaris* was only present in two cases of suppuration due to a foreign body. Fungal culture was positive in 26 per cent of the samples. It seemed that fungi found in the mouth and pharynx can also be present as saprophytes in the bronchi. Penicillin and streptomycin therapy did not seem to have any particular effect on the bacterial and fungal flora of bronchial secretion. This may be due the fact that it is difficult to produce sufficiently high concentrations of these antibiotics in the bronchi. The number of leukocytes present in the bronchial secretion might be of significance in assessing the violence of the infection and the resistance of the human organism. The writers also carried out comparisons between the bacterial flora of the bronchial secretion and that of sputum from samples taken simultaneously. Only in one-third of the cases did both florae contain the same bacteria. The flora of the sputum was considerably richer in species.

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FROM THE RESEARCH LABORATORIES, MEDICA LTD., HELSINKI

A MULTIPLE ZONE PHENOMENON IN AGAR PLATE ASSAYS WITH A VITAMIN B_{12}-REQUIRING MUTANT OF *ESCHERICHIA COLI*

by

NILS OKER-BLOM, JACOBUS SUNDMAN, and GUSTAF ÖSTLING

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Assays of Vitamin B_{12} and related compounds, especially in liver extracts by using different lactic acid bacilli in fluid media, have been performed in these laboratories for several years (4, 5, 6). These methods are, however, laborious. Attempts to assay Vitamin B_{12} with lactic acid bacilli on agar plates, which are used routinely in these laboratories in assaying other vitamins, did not give satisfactory results. When, therefore, a method of assaying Vitamin B_{12} with a Vitamin B_{12}-requiring mutant of *E. coli* (1) using the plate method in a simple medium (2) was described attempts were made to introduce this method instead of the more laborious assay with lactic acid bacilli in fluid media.

This method, as far as assay of pure Vitamin B_{12} is concerned, has given very satisfactory results with us. On testing the liver extracts, however, there appeared after one day of incubation two different zones of stimulation of the growth (Fig. 1) and, when the plates were incubated further, zones of stimulation alternating with zones of inhibition (Fig. 2). This complex pattern of the stimulation of *E. coli* by liver extract was assumed to be due to several growth-stimulating factors in the liver extract, probably with different diffusion rates, since Östling and Nyberg have shown that liver extracts contain several factors promoting the growth of *Lactobacillus leichmannii* (6). It was soon observed, however, that

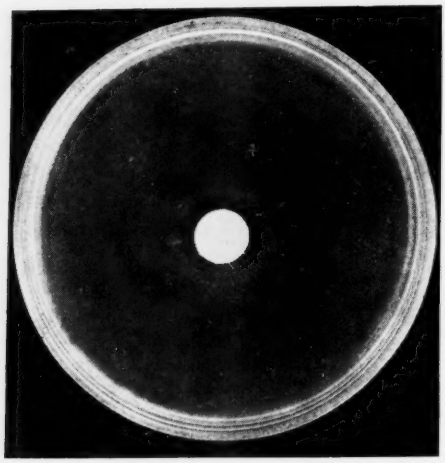

Fig. 1. — Growth of *E. coli* on the assay plate with liver extract, 24 h.

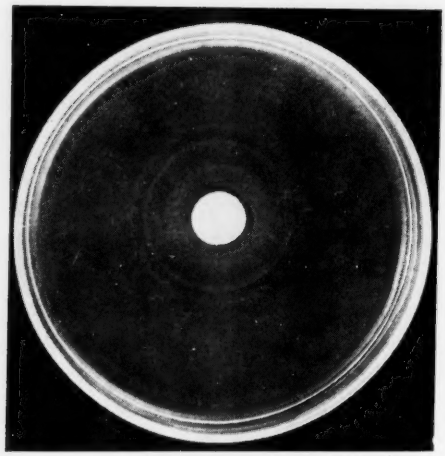

Fig. 2. — Growth with liver extract, 48 h.

zones of stimulation and inhibition were also obtained with pure Vitamin B_{12} preparations if more concentrated solutions were used than are recommended for the routine assay of Vitamin B_{12} and if the plates were incubated for 48 (Fig. 4) instead of 24 hours.

Thus the phenomenon described was, it seems, not due only to the assumed occurrence of different growth stimulating factors in the liver extract but apparently also to some other mechanism. This

paper deals with some observations made during attempts to elucidate the nature of this »multiple zone phenomenon» as far as Vitamin B_{12} is concerned.

MATERIALS AND METHODS

The B_{12} preparation employed was Vibecon Medica.

The liver extract was that used by Östling and Nyberg (6).

E. coli strain 113—3 of Davis and Mingioli (1), kindly supplied by Dr. N. Didıng of the State Bacteriological Laboratory, Stockholm, Sweden, was used in all the experiments.

The tests were made on a solid medium of the following composition: K_2HPO_4 7.0; KH_2PO_4 3.0; Na—citrate · 3 H_2O 0.5; $MgSO_4 \cdot 7\ H_2O$ 0.1; $(NH_4)_2SO_4$ 1.0; glucose (autoclaved separately) 10.0; agar 15.0; H_2O 1000.0; pH 7.0 (Medium A).

For some experiments a medium containing only one tenth of the buffering phosphates but in other respects identical with Medium A was used (Medium B).

In the experiments where an indicator was used 0.02 per cent of an alcoholic solution of Bromcresol purple was added.

From these media the assay plates were prepared in the following manner: The *E. coli* strain was subcultured in tubes each containing 5 ml of ordinary nutrient broth with one per cent Difco peptone and 0.5 per cent sodium chloride, pH 7.2. The culture was incubated at 37° C for 20 hours. After centrifugation the sediment was washed once in sterile saline and resuspended in 5 ml of saline. This amount of bacterial suspension was seeded into 150 ml of the melten agar medium, which was then poured into petri dishes in amounts of 15 ml. The assay plates were usually used immediately; occasionally they were stored in the refrigerator for one to two days before use. No difference was observed between fresh and stored assay plates.

The tests were performed in the following manner: Sterilised paper discs 13 mm in diameter were moistened with 0.05 ml of the test solution. One disc was placed on each assay plate. For each concentration of test fluid three to five plates were used.

The plates were incubated at 37° C for 1 to 96 hours, depending on the experiment.

The pH of the agar after growth was estimated in the following

manner: the different zones of stimulation and inhibition were cut out with a knife and dissolved at about 70° C in ten times their weight of distilled water. The pH was then estimated from the cooled fluid electrometrically.

In the experiments where the Vitamin B_{12} concentrations of the agar at different distances from the Vitamin B_{12} source were estimated, small agar cylinders 5 mm in diameter were stamped and tested for Vitamin B_{12} content by spacing them out on the assay plates and measuring the induced zone of stimulation.

RESULTS

Vitamin B_{12} Concentration and the Multiple Zone Phenomenon. — As mentioned, the concentrations of Vitamin B_{12} used in the routine tests (0.005—0.5 μg/ml) never, even after prolonged incubation, induced secondary zones of stimulation. These secondary growth zones, separated from the primary zone of stimulation by a zone of inhibition, did not appear until the concentration of Vitamin B_{12} was increased to about twenty times that of the concentration used in the routine tests (Table I), and then not until after 48 hours of incubation (Fig. 3 and 4).

TABLE 1

CORRELATION BETWEEN THE CONCENTRATION OF VITAMIN B_{12} AND THE EXTERIOR DIAMETER OF THE ZONES OF GROWTH AND INHIBITION

Time of Incubation		Vitamin B_{12} in μg per ml of Test Fluid					
		4	8	12	16	20	100
24 h	Primary growth zone in mm	29.4	33.1	35.5	35.5	37.4	44.4
48 h	Primary growth zone in mm	29.4	33.1	35.5	35.5	37.4	44.4
	Zone of inhibition in mm	—	—	50.5	46.4	44.3	48.6
	Secondary growth zone in mm	—	—	60.1	55.0	51.5	59.0

By stamping agar pieces from the different zones of stimulation and inhibition and placing them on new assay plates (Medium A) it was shown in several experiments that agar pieces from all the zones, including that showing inhibition of the growth, stimulated the growth of *E. coli*. The growth-stimulating effect was inversely proportional to the distance from the Vitamin B_{12} source.

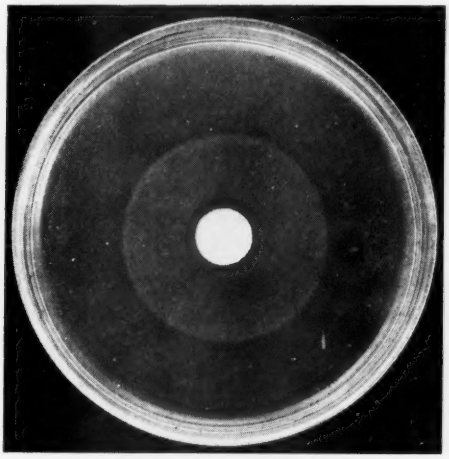

Fig. 3. — Growth with 100 μg/ml Vitamin B_{12}, 24 h (only one growth zone).

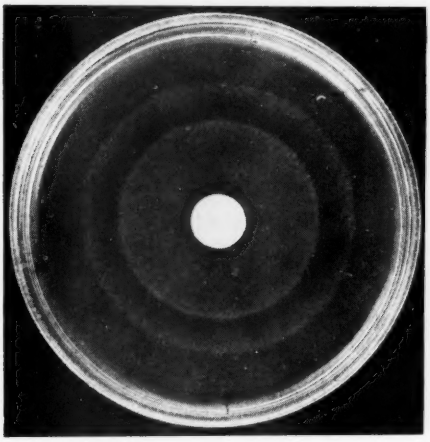

Fig. 4. — Growth with 100 μg/ml Vitamin B_{12}, 48 h (two growth zones separated by a zone of inhibition).

The Diffusion Rate of Vitamin B_{12} in Uninfected Agar. — On the basis of the previous experiments, it was of interest to know the diffusion rate of Vitamin B_{12} in the agar. Paper discs moistened in a solution containing 100 μg/ml of Vitamin B_{12} were therefore placed on uninfected plates (Medium A). Pieces of agar were stamped at one to twelve hour intervals at different distances from the Vitamin B_{12} source and placed on assay plates (Medium A). From the

experiments it was apparent that Vitamin B_{12} had spread during the first 5 hours over an area corresponding to the primary growth zone. At this time also the first visible growth of *E. coli* appeared. In the following hour Vitamin B_{12} was present also in the area of the usual zone of inhibition, and from the 24th hour in the area which after 48 hours showed a secondary growth zone. In 48 hours Vitamin B_{12} was also present in measurable amounts in the outer area of the agar.

From the two last-mentioned experiments it is reasonable to assume that in the assay plates Vitamin B_{12} was present both in the zone of inhibition and of stimulation, and that the second zone of stimulation is due to Vitamin B_{12} that failed to reach this area until about 24 hours after it appeared in the primary growth zone. This does not, however, explain the occurrence of the separating zone of inhibition.

Two hypotheses were advanced as possible explanations of this phenomenon. Because Vitamin B_{12} appeared later in the outer parts of the assay plates, which means that *E. coli* in these areas was incubated for a considerable time without Vitamin B_{12}, it was assumed that the bacteria in this area could not use Vitamin B_{12} when they got it. In the following zone, however, some hours later, they were probably again in a growth phase where they were able to use the vitamin. The second possibility is that the bacteria growing in the primary zone of stimulation produced acid enough to inhibit the growth of *E. coli* in the area of inhibition. This acid will be neutralised in the outer zone by the buffer, thus again permitting growth of the *E. coli*.

Correlation between Vitamin B_{12} and the Growth Phase of E. coli. — To investigate the ability of *E. coli* to use Vitamin B_{12} in its possible different growth phases, assay plates without Vitamin B_{12} were incubated for different times at 37° C. A paper disc moistened in 100 µg/ml of Vitamin B_{12} was then placed on the agar and the plates incubated further. Incubation of *E. coli* up to 6 hours without Vitamin B_{12} did not appreciably affect the results.

After a longer time of incubation, however, the primary zone of stimulation was considerably larger and less well demarcated than in the previous not incubated controls, until after 96 hours of incubation no growth whatsoever appeared.

This experiment thus showed that previous incubation of the

bacteria produced larger primary growth zones but did not solve the problem of the inhibition zone. Incubation without Vitamin B_{12} during the first 48 hours apparently did not appreciably diminish the faculty of *E. coli* to use Vitamin B_{12}.

Effect of Acid on the Growth of E. coli. — The effect of acid produced by *E. coli* in the primary stimulating zone on the growth of *E. coli* in the other zones was investigated in different ways:

(a) Parallelly to Medium A, Medium B was introduced, and an indicator added to both media. These experiments showed that as soon as the primary zone of growth was established there was a diffusion of acid into the outer zone. This zone of acid corresponded very closely to the usual zone of inhibition. In the cases where Medium B was used both the acid zone and the zone of inhibition were considerably larger than with Medium A, apparently due to the smaller amount of buffering substances in Medium B.

(b) The pH of Medium A was estimated during the growth at different intervals and at different distances from the Vitamin B_{12} source. The results of these pH estimations are shown in Fig. 5. As soon as the growth of *E. coli* is established in the primary growth

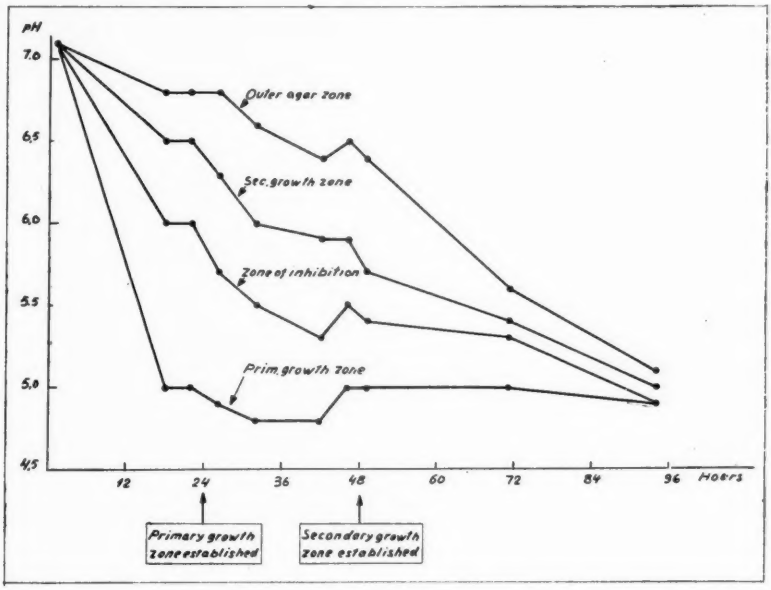

Fig. 5. — The pH values in the different zones of stimulation and inhibition during incubation with 100 μg/ml of Vitamin B_{12}.

zone the pH in this zone decreases rapidly. Some hours later a drop in the pH also appears in the usual zone of inhibition due to the diffusion of acid and at the time Vitamin B_{12} appears in this zone in measurable quantities the pH of the zone is 5.5 or somewhat less. In the secondary growth zone the pH does not decrease below 6.0 until after growth has occurred in this zone. After 96 hours of incubation the pH in the whole medium is about 5.0 or somewhat less.

(c) *E. coli* in Medium A of different pH containing a paper disc moistened in a solution of 100 μg/ml of Vitamin B_{12} showed good growth in media of Ph 6.0—7.2 and the usual zone phenomenon occurred. In the media with pH 5.0—6.0 no growth at all or very poor growth appeared and no zone phenomenon could be observed.

If pieces of agar were stamped from these plates, however, and placed on new assay plates (Medium A) the same growth-stimulating effect of the agar pieces occurred as has been shown previously, indicating that the Vitamin B_{12} content was not impaired by the pH in question.

CONCLUSION

Multiple zone formation in agar media has been observed and discussed (3, 7, 8, 9, 10, 11, 12, 13). We have not, however, been able to find any reports on the phenomenon described in this paper.

The experiments presented show clearly, in our opinion, that the cause of the multiple zone phenomenon described lies in the acid produced by *E. coli* during growth. The mechanism involved could be outlined in the following manner. If small amounts of Vitamin B_{12} are added a well demarcated zone of stimulation occurs and no multiple zone phenomenon appears. This because the acid produced does not permit the growth of *E. coli* in the neighbourhood of the primary growth zone in spite of the presence of small amounts of Vitamin B_{12}. But Vitamin B_{12} does not diffuse in the medium far enough from the source to permit secondary growth of *E. coli* in an area with favourable pH conditions. If, on the contrary, large amounts of Vitamin B_{12} are used, there will be an excess of Vitamin B_{12} which will diffuse further than the acid produced, thus allowing growth of *E. coli* outside the primary growth zone in an area where the pH conditions are still favourable.

The question of the influence of the growth phase of *E. coli* on the zone phenomenon has not been subjected to further studies. The explanation outlined above seems to give a simple answer to the problem.

If complex substances are assayed by the plate method the possibility of a single substance producing the multiple zone phenomenon should be kept in mind.

SUMMARY

A multiple zone phenomenon occurring while assaying high concentrations of Vitamin B_{12} with a Vitamin B_{12}-requiring mutant of *Escherichia coli* by the plate method is described.

The multiple zone phenomenon, consisting of a primary growth zone around the Vitamin B_{12} source and successive zones of inhibition and stimulation, appears if the plates are incubated for a prolonged period (Fig. 4). The phenomenon is apparently due to the fact that *E. coli* produces acid enough in the primary growth zone to inhibit the growth of the bacteria in the neighbourhood, although a sufficient amount of Vitamin B_{12} is present. In the following growth zone, however, the acid is neutralised by the buffering capacity of the medium, thus again allowing growth of *E. coli*.

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FROM THE DEPARTMENT OF ANATOMY, UNIVERSITY OF HELSINKI

EFFECT OF GIANT DOSES OF INSULIN ON THE CELLS OF THE PITUITARY BODY OF A RABBIT

by

ESKO K. NÄÄTÄNEN

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In a previous work (1), I have described the paradoxical effect of giant intravenous doses of insulin on rabbits. It was found that many rabbits could endure enormous doses of insulin astoundingly well. It seems that a small dose of insulin is often more effective than a very large one, reducing blood sugar more quickly and producing cramps more rapidly than a giant dose. In this experiment it was found further that this surprising phenomenon was caused by the activity of the pituitary body. The purpose of the following investigation, therefore, was to determine which of the cells of the pituitary is responsible for the said paradoxical phenomenon.

MATERIAL AND TECHNIQUE

Tests were performed on 25 male rabbits, each weighing about 2 kg. They were given from 1 to 4 intravenous insulin injections, the doses varying from 4 to 400 units per kg. When more than one injection was given, the interval between each was at least one week. The rabbits received their ordinary feed during the experiment.

The injections were given at 1 p.m. When several injections were given, the rabbit usually received a subcutaneous injection of glucose in the evening to check the effect of the insulin.

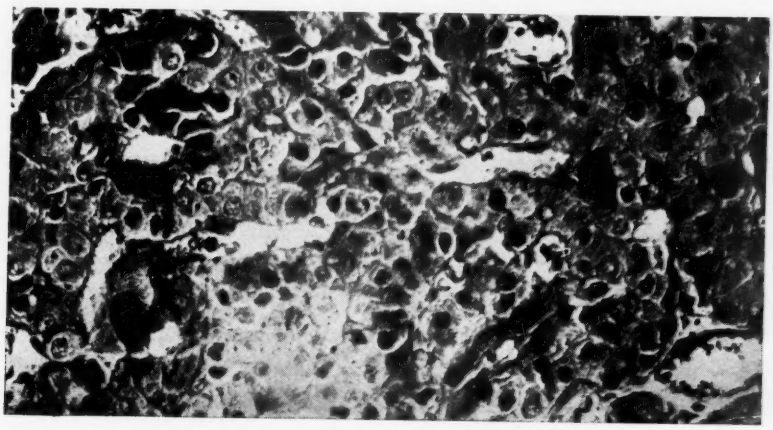

Fig. 1. — Photomicrograph showing a section of the pituitary body of rabbit No. 51, which received 400 units of insulin per kg. It was killed three hours after the injection. Darkly stained β-cells increased in number. $\times$ 500.

After the experiment the rabbit was decapitated and its pituitary removed. The specimens were then fixed in Zenker's fluid and embedded in the ordinary way. Sections 4 μ thick were stained according to Mann's long method.

Insulin used was manufactured by Medica.

RESULTS

Most of the rabbits received a very large dose of insulin, 400 units per kg, the effect of which was studied after varying intervals. For the sake of comparison, some rabbits also received doses as small as 4 units per kg. In order to determine what changes renewed insulin injections may cause in the cells of the pituitary, a few rabbits received several injections during the course of a whole month.

Since in this work attention was mainly paid to the effect of large doses of insulin in experiments lasting usually only a few hours, in my next work I shall deal with changes in the pituitary of a rat caused by renewed large insulin injections during experiments lasting for a longer period.

In some previous investigations no changes in the cells of the pituitary appear to have been caused by small doses of insulin (2). Large amounts of insulin, however, as used in my experiments,

caused several distinct changes. It is apparent that a large insulin injection causes a hypersecretion from the cells of the pituitary. Insulin acting for a short period seemed to increase the number of β-cells (the cells were not counted). The cells were often greatly enlarged and rather large, very strongly stained granules were seen. No changes in the α-cells as marked as in the β-cells could be observed; no distinct granules could be seen in these cells either.

A few days after the insulin injection the β-cells appeared to undergo degenerate changes, especially if the rabbit had received several injections. The cytoplasm would disappear and the nuclei would often become darkly stained, contracted and deformed. Similarly, sometimes the α-cells seemed to degenerate, although in a lesser degree than the β-cells.

According to the histological findings herein described, it appears that large doses of insulin are likely to produce a sharp reaction in the cells of the pituitary, especially in the β-cells. In all probability the greatly increased activity of the β-cells stimulating the suprarenal glands retards the rate of decrease in the blood sugar. On the basis of what has been stated, it would seem that the activity of the β-cells is mainly responsible for the astonishing ability of rabbits to endure giant doses of insulin.

In the description (1) of the paradoxical effect of giant intravenous insulin doses on rabbits, it was mentioned that the ability of a rabbit to withstand heavy doses of insulin deteriorates considerably if several doses are given, especially at short intervals. This phenomenon is compatible with the observation that insulin given over a long period results in the degeneration of the cells of the pituitary, especially of the β-cells.

SUMMARY

A number of male rabbits received intravenous insulin injections of different quantities, usually 400 units per kg, the purpose of this experiment being to determine what group of cells of the pituitary is responsible for the unexpected ability of rabbits to endure giant doses of insulin.

Above all, the large insulin doses seemed to affect the β-cells. Insulin injections acting for a short period seemed to increase the β-cells in number and in size. The granules of these cells were

strongly stained. Insulin injections acting for a short while usually did not cause marked changes in the α-cells. Often no distinct granules could be seen in the α-cells.

Prolonged action by insulin resulted in degenerate changes in the cells, especially in the β-cells; changes in the α-cells could seldom be observed.

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BARBAMYL
hypnoticum — sedativum

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